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6. AUTHOR(S)
Dr Gary S. Sayler

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Tennessee
Center for Environmental Biotechnology
10515 Research Drive, Suite 100
Knoxville TN 37932-2567

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AFOSR/NL
110 Duncan Ave Suite B115
Bolling AFB DC 20332-0002
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The correlation between bioavailability and biodegradative capability in the environment has always been a puzzle for bioremediation. Furthermore, the detection of biodegradative activities in situ also has hampered biological site characterization. All of these due to lack of proper tool(s) or method(s) that can be applied readily, specifically, and feasibly to the environmental pollutants. However, the development and application of bioluminescent reporter strains for continuously real-time monitoring the relationship between bacterial degradative activities and bioavailability of environmental pollutants were examined in this study. The results obtained from this investigation suggested that bioluminescent reporters can provide continuous, and precise insight information onboth molecular and physilogical level. The more important is that these bioreporters will not interrupt and complete with indigenous bacteria. The versatility of the catabolic capability on the degradation of different higher molecular PAHs by a NAH plasmid-mediated metabolism was also examined. The results obtained in this study indicated that the NAH plasmid plays an important role on the biodegradation of PAHs Furthermore, the naphthalene degradation pathways serves an essential route for the study of bacterial degradation pathway on PAHs. A

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Prepared by

Gary S. Sayler, Fu-Min Menn, Alice Layton,
Armin Heitzer, Wade Johnston, Bruce Applegate,
Yeonghee Ahn, and Lynda Callicotte

The Center for Environmental Biotechnology
The University of Tennessee
Knoxville, TN 37932
(615) 974-8080

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EXECUTIVE SUMMARY

The focus of this investigation was to develop a greater understanding of the molecular microbiology relationship to bioremediation of persistent hydrophobic organic pollutants in the environment. Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) were used as the primary models for significant classes of aromatic and chloroaromatic contaminates primarily associated with antropogenic environmental pollution. Both groups of contaminates share import similar physical-chemical environmental characteristics and represent significant environmental health hazards.

Microbiologically, two primary model systems were exploited at the molecular level to expand our understanding of the environmental relationship between biodegradative gene abundance and chemical contaminant biodegradation. These model genetic systems were the naphthalene (NAH7-like) biodegradative plasmids often associated with bacteria of the genus *Pseudomonas* and the pSS50 plasmids associated with chlorobiphenyl degradation and found commonly to occur in *Alcaligenes eutrophus*.

Investigations were thus conducted to evaluate the true environmental significance of these organisms and plasmid encoded genetic systems for biodegradation, and to test the hypotheses that 1) these genes are positively correlated with the contaminated environments, and 2) that information on environmental genetics and biochemistry of these biodegradative pathways can be extended to other chemical contaminates within each pollutant class. To conduct this research molecular tools were refined, modified and developed for more direct and specific environmental analysis. These tools included the extensive use of DNA/RNA extraction

and probing techniques, messenger RNA analysis, and *lux* gene transcriptional fusion systems to create *in situ* bioluminescent reporters of environmental biodegradation.

The results of this investigation demonstrate quantitatively that biodegradative genes associated with the well characterized naphthalene dioxygenase plasmid encoded pathway are well distributed in the environment at a level of 1% of the population in uncontaminated soils. Soil with a history of PAH contamination not only maintain much higher concentration of (100 times) of organisms harboring these genes but that their proportional abundance can increased to greater than 90% in the population due to long term selection and growth. These genes are physiological active, as measured by mRNA analysis and bioluminescent sensing, in a variety of environments comprised of mixed contaminants including jet fuels.

In addition to the environmental distribution and activity of these genes for naphthalene degradation, both new and confirmatory information was obtained demonstrating that many enzymes of the plasmid encoded genes are also responsible for degradation of other, perhaps more problematic, PAHs. These secondary substrates which may not support bacterial growth can be broken down through similar pathway. These substrates include anthracene, dibenzofuran, fluorene, phenanthrene, and dibenzo-*p*-dioxin.

For chlorobiphenyls, colony hybridization of plasmid pSS50 with other known *bph* genes exhibited a different genetic characteristic (DNA sequence) from the known biphenyl/PCB degrading bacteria. A great diversity of degradative capabilities were observed in plasmid pSS50 for degradation of PCB congeners (Aroclor 1242) and other chlorinated pesticides. These compounds include both isomers (*p,p'*- and *o,p'*-) of DDT, DDE, and DDD; and chlorobenzilate, dicofol (kelthane), hexachlorophene, etc. The results of biochemical pathways (metabolites)

study on the PAHs and chlorinated compounds indicated the existence of common degradation pathway(s) shared between bacteria for the degradation of organic pollutants.

These investigations led to major developments and improvements of *lux* gene bioluminescent reporter technology. This technology permits the development of bacterial strains which act as whole cell biosensor emitting bioluminescent light to indicate that 1) specific pollutants are present in the environment, 2) that the pollutants are bioavailable for biodegradation, and 3) that the immediate environment is permissive (not too toxic) for biodegradation to occur. In addition to *lux* sensors for naphthalene (PAHs) *lux* technology was also developed for toluene/TCE degradation and initiated for PCB degradation. In the case of the *lux* naphthalene and toluene biosensors, these organisms were shown functional in a variety of environmental matrices.

The results of this investigation can be extended to improving the predictability, performance and understanding the dynamic of natural or engineered bioremediation. New bacterial strains were also recovered that have different or alternated biochemistries for degradation of the test pollutants. These strains may permit further expansion of molecular probes for evaluating environmental biodegradation and for improving biodegradation performance in bioremediation.

1. INTRODUCTION

Microbial degradation of environmental pollutants has been studied extensively at the biochemical and molecular biological levels. Many pollutants are not of biosynthetic origin but derived from either pyrolysis of organic materials, e.g., aromatic hydrocarbons (Gibson, 1977), or are man-made chemical, e.g., chlorinated compounds. Several excellent reviews have been done during the last decade that describe microbial biodegradative capacity and mechanisms (Gibson, 1984b; Gunsalus, 1985; Timmis, 1985; Dagley, 1986; Frantz, 1986; Rochkind, 1987; Reineke, 1988; Commandeur, 1990; Smith, 1990; Chaudhry, 1991; Cerniglia, 1992; van der Meer, 1992; Furukawa, 1994a; Janssen, 1994; Layton, 1994; Singleton, 1994; Williams, 1994). Since that time a significant number of catabolic genes have been isolated and characterized from various bacteria, and have been reviewed previously (Sayler, 1989, 1990; Wallace, 1992). An updated list of recent studies on bacterial genes involved in degradation of environmental pollutants is given in Table 1. This table illustrates a wide variety of microorganisms participating in degradation of a broad number of environmental pollutants. The soil bacterium, *Pseudomonas*, still plays a major role on the biodegradation task among the known bacteria.

The biodegradation of naphthalene has been well studied; both the biochemical pathway and genetic characterization among the polycyclic aromatic hydrocarbons (PAHs) and has been thoroughly reviewed previously (Gibson, 1984b; Yen, 1988; Schell, 1990). The strain *Pseudomonas putida* PpG7 contains the NAH7 plasmid that encodes the genes for the degradative pathway of naphthalene (Figure 1). The catabolic genes in the NAH7 plasmid are organized into two operons, *nah* and *sal*, which are controlled by a positive regulator gene, *nahR*. It is important to note that the induction of these operons is controlled by the metabolite, salicylate, and by the product of the regulatory gene (*nahR*; Yen, 1982; 1985).

Table 1. Environmental pollutants for which the microbiological and molecular basis of biodegradation is well established.

Pollutant(s)	Gene	Plasmid/Chromosome encoded	Strain	Reference
Alkane (C_6-C_{10})	*alk*	Plasmid (OCT)	*Pseudomonas putida*	(Fennewald, 1979; Harder, 1986)
Alkane (up to C_8)	*amo*	-	*Nitrosomonas europaea*	(Hyman, 1988)
Alkene (up to C_5)	*amo*	-	*Nitrosomonas europaea*	(Hyman, 1988)
Aniline	*amo*	-	*Nitrosomonas europaea*	(McTavish, 1993; Keener, 1994)
Anthracene	*nah*	Plasmid (pKA1)	*Pseudomonas fluorescens* 5R	(Menn, 1993; Sanseverino, 1993a)
Benzene	n.d.	Plasmid (pWW174)	*Acinectobacter calcoaceticus* RJE74	(Winstanley, 1987)
	n.d.	Chromosome	*Bacillus stearothermophilus* BR325	(Natarajan, 1994)
	amo	-	*Nitrosomonas europaea*	(Hyman, 1985)
	bed	Plasmid (pHMT112)	*Pseudomonas putida* ML2	(Tan, 1993a; 1993b)
Benzoate	*ben*	Chromosome	*Acinetobacter calcoaceticus*	(Neidle, 1987)
	cat	Chromosome	*Acinetobacter calcoaceticus*	(Shanley, 1986; Neidle, 1988)
	pca	Chromosome	*Pseudomonas putida*	(Doten, 1987; Parales, 1993)
Benzoate(3-Cl-)	*cba*	Plasmid (pBRC60)	*Alcaligenes* sp. BR60	(Nakatsu, 1993)
	tfdCDE	Chromosome/Plasmid (pJP4)	*Alcaligenes eutrophus* JMP134	(Don, 1985b; Perkins, 1990)
	clc	Plasmid (pAC27)	*Pseudomonas putida*	(Frantz, 1987; Coco, 1993)
Benzoate(4-Cl-)	n.d.	Plasmid (pASU1)	*Arthrobacter* sp. SU	(Schmitz, 1992)
	n.d.	Chromosome	*Pseudomonas* sp. CBS3	(Scholten, 1991)
Biphenyl/4-CB	*bph*	Chromosome	*Alcaligenes eutrophus* A5	(Springael,1993)
	bph	Plasmid (pWW100)	*Pseudomonas* sp. CB406	(Lloyd-Jones, 1994)
Carbon monoxide	*amo*	-	*Nitrosomonas europaea*	(Jones, 1983)
2,4-D	*tfd*	Plasmid (pJP4)	*Alcaligenes eutrophus* JMP134	(Don, 1985a; Perkins, 1990)
	n.d.	Plasmid (pKA2)	*Alcaligenes paradoxus* 2811P	(Ka, 1994b)
	n.d.	Plasmid (pKA4)	*Alcaligenes pickettii* 712	(Ka, 1994b)
Dibenzo-*p*-dioxin	*dbf*	-	*Sphingomonas* sp. RW1	(Happe, 1993)
Dibenzofuran	*dbf*	-	*Sphingomonas* sp. RW1	(Happe, 1993)
Dibenzothiophene	*dox*	Plasmid	*Pseudomonas* sp. C18	(Denome,1993)
	n.d.	Plasmid	*Pseudomonas alcaligenes* DBT2	(Monticello,1985)
DCE(1,2-)	*dhl*	Plasmid (pXAU1)	*Xanthobacter autotrophicus* GJ10	(Tardif, 1991; Pries, 1994)
DDT	*bph*	Chromosome	*Alcaligenes eutrophus* A5	(Nadeau,1994)
Ethylbenzene	*amo*	-	*Nitrosomonas europaea*	(McTavish, 1993; Keener, 1994)
	tbu	Chromosome	*Pseudomonas pickettii* PKO1	(Olsen,1994)
Furan	*thd*	Chromosome	*Escherichia coli* NAR30	(Alam, 1990)

Naphthalene	*dox*	Plasmid	*Pseudomonas* sp. C18	(Denome, 1993)
	nah	Plasmid (pKA1)	*Pseudomonas fluorescens* 5R	(King, 1990)
	bph	Chromosome	*Pseudomonas paucimobilis* Q1	(Kuhm, 1991)
	nah	Plasmid (Nah7)	*Pseudomonas putida* PpG7	(Dunn, 1973)
	nah	Plasmid (pDTG1)	*Pseudomonas putida* NCIB9816-4	(Serdar, 1989)
	pah	Chromosome	*Pseudomonas putida* OUS82	(Kiyohara, 1994)
Octane	*alk*	Plasmid (OCT)	*Pseudomonas oleovorans*	(Chakrabarty, 1973)
Phenanthrene	*dox*	Plasmid	*Pseudomonas* sp. C18	(Denome, 1993)
	nah	Plasmid (pKA1)	*Pseudomonas fluorescens* 5R	(Menn, 1993; Sanseverino, 1993a)
	pah	Chromosome	*Pseudomonas putida* OUS82	(Kiyohara, 1994)
Phenol	*phl*	Chromosome	*Alcaligenes eutrophus* JMP134	(Pieper, 1989; Kim 1994)
	amo	-	*Nitrosomonas europaea*	(Hyman, 1985)
	dmp	Plasmid (pVI150)	*Pseudomonas* sp. CF600	(Shingler, 1992; Powlowski, 1994)
	tom	-	*Pseudomonas cepacia* G4	(Nelson 1987; 1988)
	tbu	Chromosome	*Pseudomonas pickettii* PKO1	(Kukor, 1990)
PCB	*bph*	Chromosome	*Alcaligenes eutrophus* H850	(Yates, 1989)
	bph	Chromosome	*Arthrobacter* sp. M5	(Peloquin, 1993)
	bph	Chromosome	*Pseudomonas* sp. ENV307	(Sharma, 1991)
	bph	Chromosome	*Pseudomonas* sp. KKS102	(Kimbara, 1989; Kikuchi, 1994a, 1994b)
	bph	Chromosome	*Pseudomonas* sp. LB400	(Mondello, 1989; Erickson, 1992)
	bph	Chromosome	*Pseudomonas paucimobilis* Q1	(Taira, 1988)
	bph	Chromosome	*Pseudomonas pseudoalcaligenes* KF707	(Taira, 1992)
	bph	Chromosome	*Pseudomonas putida* KF715	(Hayase, 1990)
	cbp	Chromosome	*Pseudomonas putida* OU83	(Khan, 1991)
	bph	Chromosome	*Pseudomonas testosteroni* B-356	(Ahmad, 1990; 1991)
	bph	Chromosome	*Rhodococcus globerulus* P6	(Asturias, 1994)
Protocatechuate	*pca*	Chromosome	*Acinetobacter calcoaceticus*	(Doten, 1987)
Styrene	*amo*	-	*Nitrosomonas europaea*	(McTavish, 1993; Keener, 1994)
Thiophenes	*thd*	Chromosome	*Escherichia coli* NAR30	(Alam, 1990)
Toluate (*m*-)	n.d.	Chromosome/Plasmid (pTDN1)	*Pseudomonas putida* UCC22	(Saint, 1990)
Toluene	*amo*	-	*Nitrosomonas europaea*	(McTavish, 1993; Keener, 1994)
	tom	-	*Pseudomonas cepacia* G4	(Shields, 1991)
	tmo	Chromosome	*Pseudomonas mendocina* KR1	(Yen, 1991)
	tbu	Chromosome	*Pseudomonas pickettii* PKO1	(Olsen,1994)
	tod	Chromosome	*Pseudomonas putida* F1	(Zylstra, 1988)

	n.d.	Plasmid (pDK1)	*Pseudomonas putida* HS1	(Kunz, 1981)
	xyl	Plasmid (TOL pWWO)	*Pseudomonas putida* mt-2	(Williams, 1974)
TCB (1,2,4-)	*tcb*	Plasmid (pP51)	*Pseudomonas* sp. P51	(van der Meer, 1991)
TCE	*phl/tfd*	Chromosome/Plasmid (pJP4)	*Alcaligenes eutrophus* JMP134	(Harker, 1990; Kim, 1994)
	mmo	Chromosome	*Methylococcus capsulatus* (Bath)	(Green, 1989)
	mmo	Chromosome	*Methylosinus trichosporium* OB3b	(Oldenhuis, 1989; Tsien, 1989)
	n.d.	-	*Methylomonas* GJ6	(Oldenhuis, 1993)
	tomA	-	*Pseudomonas cepacia* G4	(Nelson, 1987; Shields, 1991; 1992)
	tmo	Chromosome	*Pseudomonas mendocina* KR1	(Winter, 1989)
	tbuABC	Chromosome	*Pseudomonas pickettii* PKO1	(Leahy, 1994; Olsen,1994)
	todC1C2BA	Chromosome	*Pseudomonas putida* F1	(Wackett, 1988; Zylstra, 1989; Li, 1992)
	n.d.	Plasmid (pBD2)	*Rhodococcus erythropolis* BD2	(Dabrock, 1994)
TMB (1,2,4-)	n.d.	Plasmid (pDK1)	*Pseudomonas putida* HS1	(Kunz, 1981)
Xylene	n.d.	Plasmid (XYL)	*Pseudomonas* Pxy	(Friello, 1976)
Xylene (*o*-)	*tbu*	Chromosome	*Pseudomonas pickettii* PKO1	(Olsen,1994)
Xylene (*m*-)	n.d.	Plasmid (pKJ1)	*Pseudomonas* sp. TAB	(Yano, 1980)
	tbu	Chromosome	*Pseudomonas pickettii* PKO1	(Olsen,1994)
	xyl	Plasmid (TOL)	*Pseudomonas putida* mt-2	(Worsey, 1975)
	n.d.	Plasmid (pDK1)	*Pseudomonas putida* HS1	(Kunz, 1981)
Xylene (*p*-)	*amo*	-	*Nitrosomonas europaea*	(McTavish, 1993; Keener, 1994)
	n.d.	Plasmid (pKJ1)	*Pseudomonas* sp. TAB	(Yano, 1980)
	tbu	Chromosome	*Pseudomonas pickettii* PKO1	(Olsen,1994)
	xyl	Plasmid (TOL)	*Pseudomonas putida* mt-2	(Worsey, 1975)
	n.d.	Plasmid (pDK1)	*Pseudomonas putida* HS1	(Kunz, 1981)

n.d.: not determined. 4-CB: 4-chlorobiphenyl. 2,4-D: 2,4-dichlorophenoxyacetic acid. DCE: 1,2-dichloroethane. DDT: 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane. PCB: polychlorinated biphenyl. TCB: 1,2,4-trichlorobenzene. TCE: trichloroethylene; cometabolism. TMB:1,2,4-trimethylbenzene.

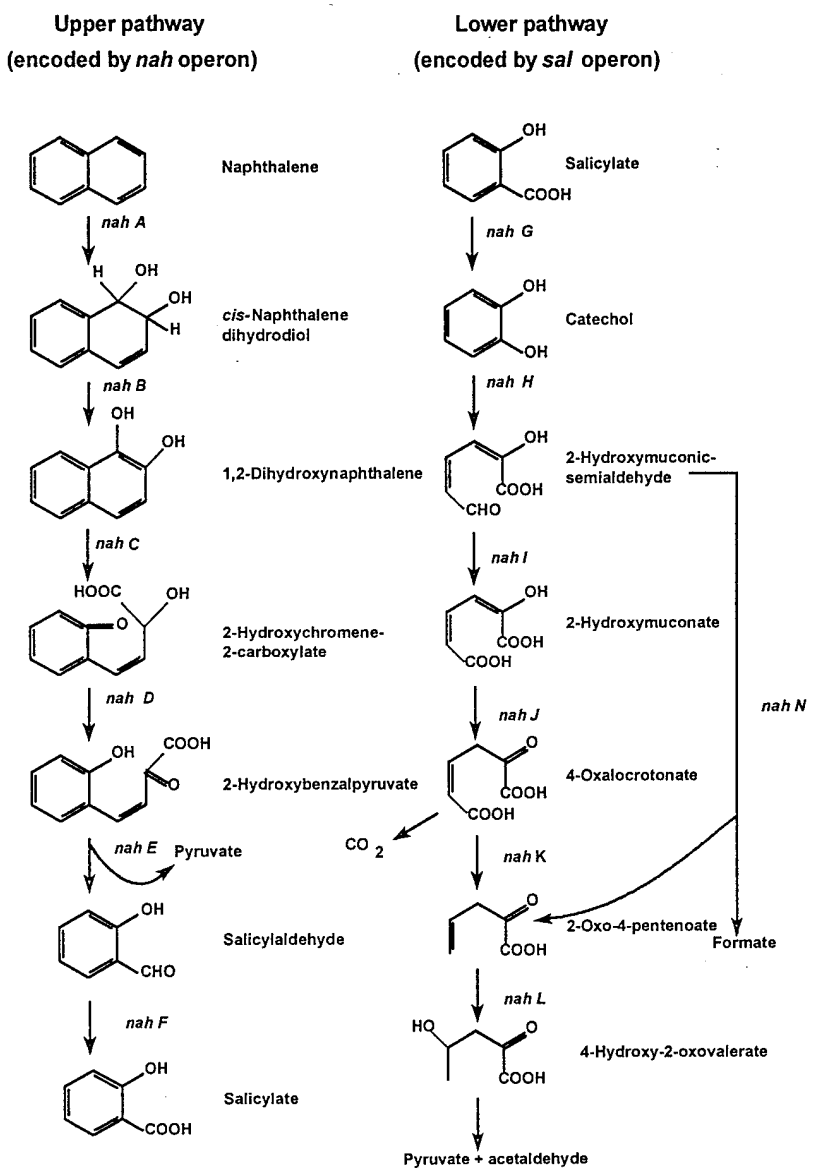

Figure 1. Naphthalene degradation pathway used by *P. putida* PpG7 encoded by the NAH7 plasmid.

Microbial degradation of other PAHs has been demonstrated for a number of bacteria (Gibson, 1984a; Dagley, 1986). It has also been demonstrated that the naphthalene plasmids, pKA1 and NAH7, mediate the catabolism of other PAHs, i.e., phenanthrene and anthracene

(Menn, 1993; Sanseverino, 1993a), and perhaps initial oxidation of some other higher molecular weight PAHs.

Bioavailability and biodegradation of pollutants are important issues in predicting the bioremediation potential of contaminated environments. Ways to continuously monitor these two processes have been difficult. While bioavailability and biodegradation of compounds can influence specific gene expression, the two tasks have often been detected *ex situ* using laboratory techniques. However, these techniques may be impractical for real-time monitoring of the two processes. Genetic manipulation of biodegradative operons has resulted in the creation of easily detectable phenotypes (reporters), that can be useful in the continuos monitoring of bioavailability and biodegradation. Such altered genes and the bacteria harboring them are called reporters and bioreporters, respectively.

Bioluminescent genes, *luxCDABE*, are classical example of reporter genes used in the genetic manipulation of other genes (Shaw, 1988). Luciferase enzyme encoded by the *lux* genes is responsible for the bioluminescent phenotype. Genetic modification of a biodegradative gene promoter by fusion of the promoterless *lux* genes result in a bioluminescent bioreporter. When expressed, the bioreporter gene produces light that can be easily detected and continuously monitored with optical devices. Bioluminescent bioreporters constructed in this manner have been investigated in laboratory conditions for the monitoring of expression of biodegradative genes.

Molecular genetic studies on the luminous marine bacteria *Vibrio fischeri*, paved the way for the development of bioluminescent bioreporters. To date, there are a handful of bioluminescent bioreporters capable of detecting specific xenobiotics and heavy metals (Table

2). The first catabolic bioreporters, *Pseudomonas fluorescens* HK44 and *Pseudomonas* sp. RB1351, were constructed employing *lux* genes to detect the bioavailability and degradation of naphthalene (King, 1990; Burlage, 1990). These bacteria produce light in the presence of either naphthalene or its catabolic intermediate, salicylate. The strain HK44 harbors a NAH7-like plasmid encoding the degradation of naphthalene to salicylate, the latter being mineralized through chromosomally encoded pathway.

Pseudomonas fluorescens HK44, a bioreporter, demonstrated the sensitivity, specificity, and applicability to detect a particular pollutant in environmental conditions at low concentrations. It was able to detect 45 ppb of naphthalene, which may not be the lower limit (Heitzer, 1992; 1994). In the studies conducted in bioreactors, the strain also exhibited the rapidity and inducibility of the luciferase enzyme system to response naphthalene concentration when perturbed feed method was applied (King, 1990). These studies ascertained the potential scope of application of HK44 in environmental systems requiring *in situ* or on-line monitoring of naphthalene bioavailability and biodegradation.

The bioluminescent bacteria are potential candidates for biosenor technology. In conjunction with sensitive, powerful, light amplifying and light collecting devices, the bacteria can be deployed in the 1) rapid detection of gene expression, 2) monitoring of bioavailability and biodegradation of pollutants, 3) investigation of factors affecting environmental processes, 4) and to study the fate and impact of genetically modified microorganisms released into the environment.

This study was a continuation work of a previous research grant, molecular ecology of bacterial populations in environmental hazardous chemical control (USAF Contract # F49620-89-C-0023). The general goals for this investigation were to develop new bioluminescent

Table 2. Chracaterization of known bioreporters.

TYPE / BRAND OR GENERIC TERM	DETECTION METHOD	PURPOSE	MICROORGANISM/ PLASMID	REFERENCE
SPECIFIC BIOSENSORS				
nah-lux	Bioluminescence	Detect naphthalene and salicylate	*Pseudomonas fluorescens*, pUTK21; *Pseudomonas* sp., pUTK9	(King, 1990, Burlage, 1990)
bph-lux	Bioluminescence	Detect PCBs	*Alcaligenes sp* A5	(Springael, 1991)
xyl-lux	Bioluminescence	Detect xylene	*Pseudomonas putida* pUTK24	(Burlage, 1992)
tod-lux	Bioluminescence	Detect toluene, and TCE degradation	*Pseudomonas putida* B2	(Applegate, unpublished data)
pvd-inaZ	Ice nucleation	Detect iron	*P. fluorescens, P.syringae*	(Loper, 1994)
mer-lux	Bioluminescence	Bioavailable mercuric ions	*E.coli*; pRB28, pOS14, pOS15	(Selifonova, 1993)
cup-lux	Bioluminescence	Detect copper	*Alcaligenes eutrophus* DS185; AE984	(Corbisier, 1992, 1993a)
czc-lux	Bioluminescence	Detect zinc	*A. eutrophus*	(Corbisier, 1992)
cnr-lux	Bioluminescence	Detect nickel and cobalt		(Corbisier, 1992)
chr-lux	Bioluminescence	Detect chromium		(Corbisier, 1992)
thl-lux	Bioluminescence	Detect thallium		(Corbisier, 1992)
cad-lux	Bioluminescence	Detetct arsenic and cadmium	*Staphylococcus aureus*; pI258	(Corbisier, 1993b)
NON-SPECIFIC BIOSENSORS				
RODTOX^{R}	Respiration	Rapid BOD	Native microbial community	(Van Rolleghem, 1990)
MICROTOX^{R}	Bioluminescence	Toxicity test	*Photobacterium phosphoreum*	(Beckman Instruments, 1978 de Zwart, 1983)
TOXI-Chromotest	Inhibition of de novo synthesis	Antibiotics and Toxins test	*Escherichia coli*	(Environmental Biodetection Products Inc., Ontario, Canada)
MetPad^{TM}	Enzyme Inhibition, β-galactosidase activity	Heavy metals: Cd, Cu, Pb, Hg, Ni and Zn ions	*E.coli*	(Bitton, 1992; de Vevey, 1993)
Ames test	Mutagenesis	Genotoxicity	*Salmonella typhimurium*	(Ames, 1971, 1972; Maron, 1983)
Mutatox^{TM}	Mutagenesis / Bioluminescence	Genotoxicity	*Vibrio fischeri* M169	(Microbics reference D006)

reporter bacterial strains and to apply these molecular tools to determine optimal and sustainable biodegradation of environmental pollutants under field or bioreactor conductors. In addition, this study was also focused on the NAH degradation system and use as a primarily model for studying the degradation of other higher molecular weight PAHs. The biodegradation of chlorinated compounds was also examined in current work. The specific objectives of this investigation were described as:

(1) Continuation of development new bioluminescent reporter strains for real-time monitoring bioavailability and *in situ* bioremediation process(s) of environmental pollutants.

(2) Determination of correlation between light induction and biodegradative capabilities in the bioluminescent reporter system.

(3) Evaluation of the molecular and environmental stability of genetically-engineered microorganisms (GEMs) - bioreporter strain..

(4) Elucidation of the diversity of bacterial degradative capabilities on the degradation of PAHs and chlorinated compounds by a NAH plasmid and a *bph* genes, respectively.

(5) Illustration of a diverse naphthalene catabolic pathway by *Burkholderia cepacia* strain JS150.

2. MATERIALS AND METHODS

2.1 Bacteria and Media. The following isolates, 5R, 5RL, DFC49, DFC50, HK44, and A5 were isolated previously and selected to be used in the study. Two strains, PpG7 and

PB2440, were used in the control experiment. The properties of these strains are summarized in the Table 3.

Table 3. Characteristic of selected bacterial strains used in more detailed analysis for this study.

Strains	Plasmid	M.W.	Phenotype
E.coli DF1020	pRK2013	–	Amp^R, Km^R, Tra^+
E. coli DH5	pDTG514	2.7kb	Amp^R
E. coli HB101	pUCD615	–	Amp^R, Km^R
A. eutrophus A5	pSS50	50.9 kb	$4\text{-}CB^-$,$4\text{-}CBA^+$
P. fluorescens 5R	pKA1	101 kb	Nah^+,Phe^+,Ant^+
P. fluorescens 5RL	pUTK21	116 kb	Nah^+,Sal^-
P. fluorescens 18H	multiple	–	PSI^-, Nah^-,Sal^+
P. fluorescens HK44	pUTK21	116 kb	Nah^+,Sal^+
P. putida B2	pUTK30	–	Amp^R, Km^R, Tod^+, Lux^+
P. putida F1	N/A	–	Tod^+
P. putida G7	NAH7	83 kb	Nah^+,Phe^+,Ant^+
P. putida PB2440	N/A	N/A	PSI^-, Nah^-,Phe^-,Ant^-
P. putida RB1351	pUTK9	–	PSI^-, Nah^+,Sal^+, Lux^+

Nah: naphthalene; Phe: phenanthrene; Ant: anthracene; Sal: salicylate; 4-CB: 4-chlorobiphenyl; 4-CBA: 4-chlorobenzoate.
N/A: Not applicable.

Bacteria were routinely isolated and cultivated on a full or 1/4 strength yeast extract-peptone-glucose agar (YEPG) medium containing (g/L): 0.2 of yeast extract, 2.0 of polypeptone, 1.0 of glucose and 0.2 of NH_4NO_3 (pH 7.0). Additional complex media used included: yeast extract-peptone-salicylate-succinate medium (YEPSS, used for inducing naphthalene degrading bacteria); Luria broth (LB) medium, and Pseudomonas isolation medium (Difco). Agar plates were made by adding 1.5% (w/v) Bacto-agar (Difco) and antibiotics were included as necessary. A minimal medium for sole carbon growth studies was also used. The minimal medium consisted of (g/l): KH_2PO_4, 0.68; K_2HPO_4, 1.73; $MgSO_4.7H_2O$, 0.1; NH_4NO_3, 1.0, and 0.1 ml of

a mineral salts solution (20). Mineral salts buffer (pH 7.0) used in the plate count procedure was composed of (g/l): $NaNO_3$, 4.0; KH_2PO_4, 1.5; $FeCl_3.6H_2O$, 0.005; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.01; Na_2HPO_4, 0.5.

The minimal salts medium supplemented with 0.005% yeast extract (MSY) was used in the experiment for measuring ^{14}C-DDT mineralization. The minimal salts (basal salts) media consisted of 4.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.005 g $FeCl_3$, 0.2 g $MgSO_4$, 0.01 g $CaCl_2$, 0.5 g Na_2HPO_4 in 1 L distilled water (pH 7.0).

2.2 Chemicals. All chemicals used in this investigation were purchased highest grade commercial available and used without further purification unless other specified.

2.3 Soil Samples. Soils used in this investigation were original obtained from manufactured gas plant sites and were heavily contaminated with naphthalene and higher molecular weight polyaromatic hydrocarbons (PAHs). The six PAHs-contaminated soils were designated A through E, and N, and one creosote-contaminated soil was designated as G were used in this study. The characterizations of soils are listed previously (Sanseverino, 1993b). The properties of soils are described by Cushey *et al.* (1990). The Etowah and Pamlico soils were used as uncontaminated controls. All soils were stored at 4°C until used.

2.4 Phenotypic Characterization of PAH-Degraders. The bacteria in the collection were originally isolated from the soils as described in the above Section, and following methods were used for phenotypic characterization: spray plate, mineralization, and indigo test. The collection was stored at -70 °C before use.

2.4.1 PAH spray plate assay. The PAH spray plate method (Kiyohara, 1982) was employed to test the PAH degradative ability of isolates in the collection. The PAHs used in the

spray plate methods were phenanthrene, fluorene, anthracene, pyrene, and Benzo[a]pyrene. Bacterial colonies were pre-grown on YEPG agar plates and then sprayed with a 1% (w/v) PAH dissolved in acetone. After evaporating the acetone by placing the plates in the hood for 1 minute, the plates were placed in plastic bags and incubated up to 3 weeks and were observed daily. Colonies producing clear zones by the solubilization and degradation of the PAH were scored positive. *Pseudomonas putida* PB2440 (Bagdasarian, 1981) was used as a negative control. Only acetone without dissolved PAHs was used for an additional negative control.

2.4.2 Indigo test. The indigo test was used to examine dioxygenase as described previously (Ensley, 1983). Dioxygenase activity of each isolate was examined by exposure to indole vapor. Bacterial colonies were pre-grown on YEPG agar plates, and then indole crystals were placed on the lid of the petri dish. After 1 day of incubation at room temperature, colonies that produced a blue color were scored positive. *P. putida* PB2440 was used as a negative control.

2.4.3 DNA hybridization. Gene probing was used to determine if the isolates contained NAH7-like catabolic genes. All DNA hybridizations were performed as previously described (Sanseverino, 1993b, Church, 1984), and *P. putida* PB2440 and *P. putida* G7 (NAH7) (Dunn, 1973) were used as negative and positive controls, respectively. DNA was blotted on nylon filters. The filters were then baked at 80°C for 1 h and stored at room temperature until use. One buffer (0.5 M NaH_2PO_4, 1 mM EDTA, and 7% sodium dodecyl sulfate; pH 7.2) was used for prehybridization and hybridization at 65 °C. Washing of filters were carried out at 65°C.

Colony hybridization was performed using NAH7-derived gene probes, *nahA*, *nahG*, and *nahH* probes. The negative results of colony hybridization were further confirmed by isolating

the total genomic DNA from bacteria then subjected to slot blot hybridization using the same probes. To, The slot blot filter was further rehybridized with a ^{32}P-labeled 23S rRNA gene probe (Festl, 1986) to verify the availability of DNA on the filter. All procedures; prehybridization, hybridization, and washing; were performed under low-stringency conditions at 45°C.

Thermal Cycler PCR amplification (Perkin Elmer Cetus) was used to obtain NAH7-derived gene probes: *nahA* (encoding naphthalene dioxygenase), *nahG* (encoding salicylate hydroxylase), and *nahH* (encoding catechol-2,3-dioxygenase) probes. NAH7 plasmid was purified by CsCl-EtBr density gradient ultracentrifugation for probe preparation. The amplified products were then detected by using 1% agarose gel electrophoresis. Radiolabeled ^{32}P-DNA probes were generated by asymmetric amplification using only one primer to generate single stranded radiolabeled probes. ^{32}P-dCTP was used for the labeling. The labeled probes were purified using a NucTrap Push Column (Stratagene, La Jolla, Ca.). The specific activities of the probes were quantified by a Beckman model LS 5000 liquid scintillation counter. Specific activity of the labeled probes was approximately 10^8 disintegration per minutes (dpm) mg^{-1} of DNA.

2.5 Mineralization Assay. PAH mineralization was used in screening the culture collection by measuring conversion of ^{14}C-PAH to $^{14}CO_2$. All procedures used in this study were described in previously papers (Sanseverino, 1993a; 1993b). The following ^{14}C radiolabelled PAHs were used in this investigation: [U-^{14}C]anthracene (uniformly, 15.0 mCi/mmol; purity >98%); [7,10-^{14}C]benzo[a]pyrene (60 mCi/mmol; purity >98%) and [7-^{14}C]benzo[a]pyrene (58.6 mCi/mmol; purity >99%); [1-^{14}C]naphthalene (10.1 mCi/mmol; purity >98%); [9-^{14}C]phenanthrene (10.4 mCi/mmol; purity >99%); and [4,5,9,10-^{14}C]pyrene (10.4

mCi/mmol; purity >98%). All the ^{14}C-labeled PAHs were purchased from Sigma Chemical Corp. (St. Louis, MO) except ^{14}C-benzo[a]pyrene was obtained from Amersham (Arlington Heights, Ill). The experiment was carried out in 25 ml EPA vials (Pierce Chemical Co., Rockford, Ill) with teflon/silicone septa (Pierce) and 8 ml glass vials placed within the larger vials. Each strain/PAH combination was performed in triplicate plus a killed control. The killed control consisted of adding 0.5 ml of 2 N sulfuric acid at the start of the experiment. The radioactivity was measured using the scintillation counter described previously.

^{14}C-DDT mineralization experiments were performed in duplicate as described previously (Nadeau, 1994). [U-^{14}C]DDT (291 mCi/mg; purity >99.9%) was added at a final concentration of 1 ppm and was purchased from Amersham (Arlington Heights, Ill). The radiolabeled purity was further confirmed by a HPLC system equipped with a Radioactive Flow Detector (FLO-ONE\beta, Radiomatic Instruments and Chemical Co., Inc; Tampa, FL). Analysis of ^{14}C-DDT degradative products was conducted by using TLC technique. ^{14}C-DDT and metabolites analyzed by TLC were separated by a hexane:ethanol (75:25) solvent system, and detected and quantitated with a Bioscan Imaging Scanner System 2000 with AutoChanger 1000 (Bioscan, Inc., Washington, D.C.).

2.6 Maintenance and Stability of *nah-lux* Reporter Plasmids and Strains.

Continuous cultivation was performed on a New Brunswick C30 chemostat with a Teflon headplate and Teflon tubing was used for continuous cultures. Media was supplied using a FMI peristaltic pump equipped with a Teflon head (Fluid Metering, Inc.). Cultures were aerated continuously at 0.2 L/min and agitated at 250 rpm at 25°C. A buffered mineral salts media (Meynell, 1965) was used with saturating concentrations of either naphthalene or salicylate or

glucose as indicated. Bioluminescence was measured using an Oriel Model 7070 photomultiplier with a liquid light pipe. Bioluminescence data was collected on-line by an IBM PS/2 computer using acquisition software written by Rod Bunn (Biological Services, University of Tennessee).

2.6.1 Strain Monitoring. Strains were routinely monitored in the chemostat by spread plating on YEPG plate containing antibiotics. *P. putida* RB1351 was monitored on YEPG containing ampicillin and kanamycin (both 50 μg/ml). *P. fluorescens* 5Rrif numbers were determined by plating on YEPG containing rifampicin (50 μg/ml). *P. fluorescens* HK44 numbers were monitored on YEPG containing tetracycline (14 μg/ml). Culture density was monitored spectrophotometricaly by absorbance at 595 nm. The loss of plasmid pUTK9 from *P. putida* RB1351 was monitored as the difference between the colony forming units on PSI and YEPG containing ampicillin and kanamycin. Plasmid maintenance of pKA1 was monitored using YEPG agar plates supplemented with 100 μg/ml of indole as a presumptive assay for *nah*A activity (Ensley, 1983). All error bars on the graphs showing population maintenance represent one standard deviation of the mean.

2.6.2 DNA Probing. Colonies from spread plates were blotted onto Biotrans nylon filters (ICN Biomedicals, Inc.) The colonies were lysed and the DNA denatured with 0.2 N NaOH, and the filters were baked at 80°C for 1 hr. All filters were prehybridized using the method of Church and Gilbert (1984). Colony blots were probed with single stranded ^{32}P-*luxAB* specific DNA. Probe was generated using the polymerase chain reaction (PCR). Previously published sequences (Baldwin, 1989) were used to design the oligonucleotides for the sense strand 5'-GGGGGTTGTTATTCCAACAGC-3' and the opposite strand 5'-GTCATCATGAGACC-CTACTGC-3' (Genosys Biotechnologies, Inc.). The plasmid pUCD623 (Shaw, 1988) was used

to obtain vector free double stranded template DNA. This double stranded template was used along with one of the oligonucleotides and ^{32}P-αdCTP to produce single stranded probe. All hybridizations and washings were done at high stringency. Positive signals were detected using autoradiography.

2.6.3 Plasmid Isolation and Analysis. The molecular stability of reporter plasmids was determined by restriction profiles. Colonies were randomly picked from chemostat culture platings and subject to mini-prep plasmid isolation. The mini-prep procedure was a modification of the Promega protocol (Technical Bulletin #009). The resulting plasmid DNA was of sufficient purity for restriction endonuclease digestion. The plasmid preparations were treated with the endonuclease *Bam*H1. The DNA fragments were resolved on a 0.8% vertical agarose gel and visualized by staining with ethidium bromide.

2.6.4 Substrate Monitoring. Chemostat samples were analyzed for parent compound and metabolites by HPLC. A 3 ml aliquot of each sample was extracted against an equal volume of hexane-isopropanol (4:1). The extract was analyzed by HPLC (Perkin-Elmer series 410) with a C18 column using UV detection at 254 nm. The mobile phase was methanol:water (85:15) at 1.5 ml/min. This procedure could resolve the test compounds and metabolites.

2.6.5 Statistics. All error bars on graphs indicating bacterial populations represent one standard deviation of the mean of triplicate platings from duplicate dilutions series. Error bars on graphs indicating the ratio of plasmid-bearing and plasmid-free populations represent propagation of error analysis as shown in the following method,

$$\frac{s_y}{y} = \sqrt{\left(\frac{s_a}{a}\right)^2 + \left(\frac{s_b}{b}\right)^2}$$

where s_y is the absolute standard deviation, y is the mean quotient, and s_a and s_b are the relative standard deviations of the means a and b (Skoog, 1986).

2.7 Bioluminescence Assay. Bioluminescence measurements were conducted on-line as described in previously (Heitzer, 1992; 1994). Strain *P. fluorescens* HK44 was used in this study. Data were stored automatically on a personal computer for on-line measurement experiments and the software program was written by Rod Bunn (University of Tennessee). For perturbation experiments, baseline bioluminescence in the absence of an inducer was subtract prior to log transformation and further analysis data. The light output of the culture was measured in 15-min intervals with an Oriel digital display (model 7070), an Oriel photomultiplier (model 77340), and a liquid light cable. The light readouts were obtained as an amperometric signal and are presented in nanoamperes.

Salicylate and naphthalene solutions were prepared in buffered mineral salts medium and used for bioavailability study.. The experiments were conducted as two independent sets of three experiments each. For the salicylate and naphthalene standard curves, all the values were corrected for the light output of the control without an inducing substrate. The corresponding standard deviations were calculated by taking the error propagation into account.

Saturated naphthalene sterile solution (approximately 31 mg) and salicylate sterile solution (10 mg/l) were used as waste stream solutions in on-line monitoring studies. The total liquid volume of the biosensor that used in this study was 1.5 ml and described previously (Heitzer, 1994). Two HPLC pumps were used for deliver medium and waste stream solutions or water at a flow rate of 2.5 ml/min, resulting in a constant total flow rate of 5 ml/min. Experiments were conducted at a constant, controlled temperature of 25 °C and at pH 7.

2.8 Construction of *tod-lux* Reporter Strain. *Pseudomonas putida* F1 was grown on a YEPG medium at 28°C. *E. coli* transformants were screened on LB plates containing ampicillin (50 μg/ ml). Transconjugants were screened on pseudomonas isolation agar containing kanamycin (50 μg/ ml) and toluene vapor. One liter cultures of *E. coli* DH5 alpha harboring the appropriate plasmid were harvested and plasmid DNA was isolated using a modified alkaline lysis procedure (Maniatis, 1982). The plasmid DNA was then subjected to CsCl density gradient purification, followed by butanol extraction and ethanol precipitation (Maniatis, 1982). Plasmid DNA was resuspended in TE buffer (10 mM tris, 1 mM EDTA) and stored at 4°C until use. Restriction enzymes and T4 ligase were obtained from GIBCO BRL (Gaithersburg, MD) and used according to manufacturers protocols. Cloning techniques were performed as outlined in Manniatis *et al* (1982). Transformations were performed using subcloning efficiency competent cells purchased from GIBCO BRL according to manufacturers protocol. Mini preps of transformants were performed as described previously (Holmes, 1981).

Triparental matings were carried out using a modified version of the filter technique. Pure cultures of donor (JBF-7), Helper (2013), and Recipient (F-1) were grown to an OD_{546} of approximately 1.0 in LB broth with selective pressure. Cells were harvested by centrifugation at 8,000 rpm for 10 min. The pellets were resuspended and washed three times in 100 ml 50 mM KH_2PO_4, after which they were resuspended in 50 ml KH_2PO_4. A PC membrane (47mm X 1.0 um) was placed on a non-selective (LB) agar plate and the three strains were mixed using a ratio of 2:1:1 (Donor:Helper:Recipient). The mixtures were incubated overnight and the filter was then removed and washed in 1.5 ml of 50 mM KH_2PO_4. A serial dilution was carried out and dilutions were plated on LB Km^{50} plates and incubated for approximately 48 hours.

Exconjugants were then exposed to toluene vapor and those which produced light were selected for further characterization.

2.9 Co-Oxidation of TCE by *tod-lux* Reporter Strain B2. The strain B2 was grown in a liter LB broth to an OD_{600} of 1. The cells were then centrifuged at 6000xg for ten minutes and washed in 0.9% NaCl. The final wash was resuspended in 120 ml of an alginic acid solution. Next the cell-alginate suspension was placed in a 60 ml syringe, forced through a 25 gauge needle, and allowed to drop into a 0.5 M $CaCl_2$ solution. The alginate was cross linked by the Ca ions, thus encapsulating the cells. The cells were then placed in a fresh solution of $CaCl_2$ and allowed to sit for 30 minutes prior to use.

A differential volume reactor (DVR) system was designed to simulate a section of an ideal plug flow reactor. A complete description of this reactor can be found in previously paper (Webb, 1991). In this investigation, a system was designed incorporating the DVR as illustrated in Figure 2. The system was equipped with three Millipore stainless steel substrate containers rated to 690 kPa. The feed from the substrate vessels to the reactor inlet was controlled by two Gilson 301 HPLC pumps. The substrate vessels were pressurized with either oxygen or air to provide the system with an electron acceptor. All medium vessels contained trace mineral medium (Lackey, 1993; Menn, 1991) with the addition of 3 mg/L pyruvic acid and a 0.1 M solution of trizma base kept at a pH = 7.0. In addition to medium, the different vessels contained either TCE, toluene, or no extra carbon source. The reactor effluent entered a 5 inch stripping column that was packed with 3 mm glass beads to provide adequate surface area for the volatile separation process. The volatiles were stripped with helium, the GC carrier gas, in a concurrent fashion through the stripper. The outlet of the stripping column was attached directly to a gas

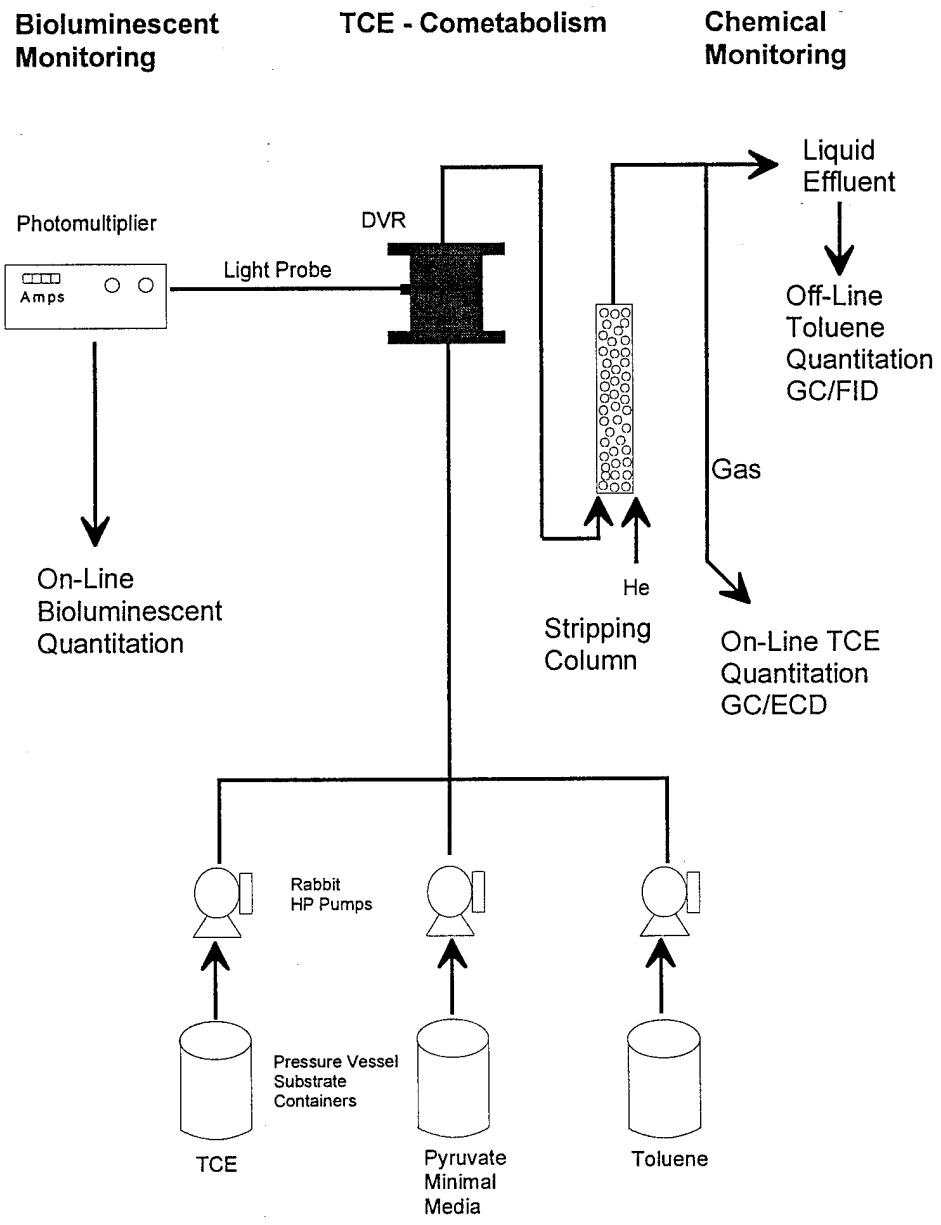

Figure 2. Schematic diagram of on-line system used to monitor the cometabolism of TCE.

chromatograph (Packard 5890 Series II) with an electron capture detector (ECD) by a heated sample line maintained at 75°C. Automatic injections (25 µl sample loop) were made on the GC by a computerized control process (Hewlett Packard computer, HP Chem Station software). The

GC was equipped with a cross-linked methyl silicone capillary column (internal diameter of 0.2 mm, 0.33 μm film thickness) while the oven temperature was operated isothermally at 60°C. Other operating parameters include an injection temperature of 150°C, the detector temperature of 200°C, and a split ratio was set at 10:1. This system was equipped with a bypass line around the reactor in order to calibrate the stripping column. Liquid sampling ports for measuring oxygen concentration were available on the inlet and outlet lines of the system. Gas sampling ports for measuring inlet and effluent toluene concentrations were available. Toluene samples were analyzed using a Schimadzu GC-9A gas chromatograph equipped with a 2.44 m, 3.2 mm diameter Poropak N packed column and a flame ionization detector. The isothermal temperature of the oven was 210°C, and both the detector and injector temperatures were 220°C. A photomultiplier and computer were used to collect the bioluminescence data.

2.10 Construction of *Biphenyl-lux* Reporter Strains. Bioluminescent reporter strains were made by inserting portions of the biphenyl operon from a PCB degrading organisms similar to *Pseudomonas* LB400 (Mondello, 1992) into a promoterless *lux* operon from *Vibrio fischeri* carried on a broad-host-range vector pUCD615 (Rogowsky, 1987). Two *bph-lux* constructs were made from the beginning region of the biphenyl operon (Figure 3) using standard molecular biology techniques (Sambrook, 1989). The first construct was made by cloning a 2.8 Kb *Eco*RI fragment from the plasmid C14-15 (Layton, 1994) containing the *bphABC* genes into pUCD615 (*ORFObphA-lux*). The second construct was made from a *Avr*II to *Nsi*I deletion of ORFO followed with a substitution with a multirestriction site fragment. From this plasmid a 1.5 Kb *Eco*RI fragment containing just the P2P1 *bphA* piece was cloned into pUCD615 (*bphA-lux*). The plasmids *ORFObphA-lux* and *bphA-lux* transferred to *Pseudomonas putida* PB2440 with and

without a transposon containing the PCB degrading genes (TnPCB; Lajoie, 1994). Strains were grown overnight in PAS minimal salts medium with 60 mM sodium pyruvate as the carbon source. The cultures were centrifuged and resuspended in PAS media to a concentration of OD_{600} of 0.6-0.7. Carbon sources were added to triplicate vials containing 5 ml bacterial cultures. The vials were incubated at 25°C with shaking (100-200 rpm). Light was measured over time using a photomultiplier. The DNA hybridization methods for the biphenyl gene probes are described in previously (Layton, 1994).

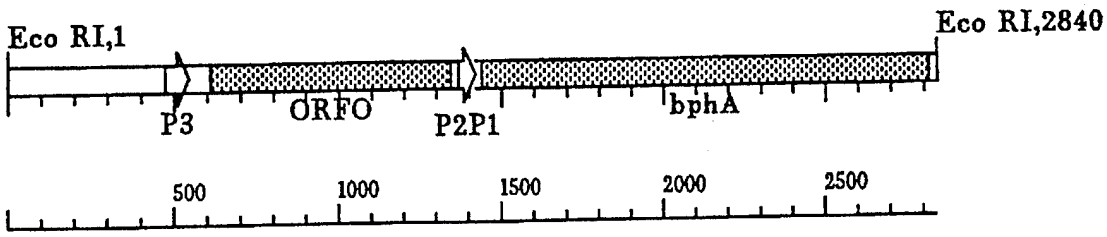

Figure 3. Map of 2.8 Kb region of biphenyl operon containing ORFO and *bphA* and promotor regions.

2.11 Biodegradation of PAHs and Chlorinated Compounds. *Pseudomonas fluorescens* 5RL exhibits a Nah^+Sal^- phenotype and used in this study. The growth condition of the bacterium and the biotransformation procedures were described previously (Menn, 1993). The following PAHs were tested in the study: anthracene, dibenzofuran, dibenzo-*p*-dioxin, fluorene, and phenanthrene. All substrates were dissolved in DMF and added between 5 - 20 mg per 100 ml of phosphate buffer (pH 7.5). Incubation of the substrates without strain 5RL are used as abiotic control.

Alcaligenes eutrophus A5 was identified and characterized as a PCB-degrader previously (Shields, 1985; Pettigrew, 1990), and was used in aerobic degradation of DDT. A 5 mM of *p,p'*-DDT was used in transformation experiment and carried out in mineral salts medium as described previously (Nadeau, 1994). Biotic controls consisted of strain A5 and DMF.

In addition, some DDT structure-like pesticides were also examined by strain A5 using the method described above. The following compounds were tested in this investigation: *o,p'*-DDT, *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDD, *p,p'*-DDD, bifenox, chlorobenzilate, dibenzo-*p*-dioxin, dicofol (kelthane), hexachlorophene, methoxychlor, and perthane.

2.11.1 Analytical Methods for Metabolite Study. The UV/Vis absorbance measurements of the supernatants from *P. fluorescens* 5RL and *A. eutrophus* A5 cultures supplemented with different substrates were performed using a Beckman DU20 spectrophotometer (Fullerton, CA). The supernatants were acidified with 2 N HCl and made to basic with 2 N NaOH. Samples were scanned over a wavelength of 200 to 600 nm at a scanning rate of 300 nm/min.

Mass spectrometric characterization of metabolites was performed on cultures incubated with and without different substrates. The metabolites were identified using a Hewlett-Packard GC/MS (Palo Alto, CA; model 5995A) or a VG Trio-3 mass spectrometer, that equipped with a Hewlett-Packard gas chromatograph (model 5890), with a 12 meter 0.25 mm BP5 capillary column consisting of 5% diphenyl dimethyl siloxane (SGE, Austin, TX). The column temperature parameters were set at an initial temperature of 50°C for 1 minute followed by a 10°C per minute increase to 250°C. The ionization voltage was -70 eV.

2.12 Resting Cell Assays for PCB Degradation. The range of PCB congener metabolism by *Alcaligenes eutrophus* A5 was determined using a 48-hour resting cell assay with

10 ppm Aroclor 1242 (Bedard, 1987). Bacterial cells were grown in PAS (Bedard, 1986) with 100 ppm to an OD_{600} of 0.7 to 1.0. The bacterial cells were collected, centrifuged and washed two times with .05M Na-phosphate buffer pH 7.5. The bacterial cells were resuspended in a .05 M Na-phosphate buffer pH 7.5 to an OD_{600} of 1.0. 3 ml of bacterial cells were placed into each of 4 sterile EPA vials with teflon caps. 2 vials of cells were killed with 30 µl of perchloric acid. After 10 minutes, 10 µl of a 3 mg/ml Aroclor 1242 stock solution was added to each vial. The vials were incubated on a rotary shaker (200 rpm) at 25°C for 2 days. The bacterial cells in the remaining 2 vials were killed with 30 µl perchloric acid. The PCBs were extracted from each vial by first adding 3 ml of ether (nanograde, Baxter; McGraw Park,Ill) and shaking for 1 hour on a reciprocating platform shaker. The vials were centrifuged for 10 minutes at 190 xg on a Beckman Model TJ-6 centrifuge. A 1 ml ether sample from the upper phase was transferred to a Teflon-capped glass tube containing 4 ml of hexane and 0.5 g silica gel (35-60 mesh). The tubes were mixed on a vortex mixer, then shaken for 15 minutes on the reciprocating shaker, and centrifuged for 10 min at 190 xg. The final ether-hexane extracts were transferred to glass vials for gas chromatograph analysis.

Relative concentrations of PCB congeners were determined using a Shimadzu (Kyoto, Japan) model GC-14A gas chromatograph equipped with an AOC-14 autosampler and an electron capture detector and split/splitless injector, both maintained at 300°C. An SPB-5 capillary column (30m x 0.25 mm, i.d.; Supelco, Inc.; Bellefonte, PA) was used with nitrogen as both the carrier (0.88 ml/min) and make-up (35 ml/min) gas. Injection of 1 µl injection was performed using the splitless mode. The column oven temperature was held at 40°C for 2 min, raised to 80°C at a rate of 10°C/min, and then to 225°C at 6°C per minute and held for 45

minutes. Individual congeners of Aroclor 1242 were quantified and identified by comparison of congener profile with those reported by Bedard *et al* (1987). The percent degradation was calculated by normalization of the peak areas to an unmetabolized reference peak (peak 41) and then by comparison of the peak areas between the live cells and the killed controls.

2.13 Plasmid-Mediated Dehalogenation of 4-Chlorobenzoic Acid. The bacterial strains *Alcaligenes* A5, *Alcaligenes* ALP83 and strain 1C1 were cultured in YEPG media or minimal salts media with 20 mM Pyruvate and 0.01% yeast extract (Layton, 1992). Plasmid DNA was isolated from these strains using an alkaline lysis procedure from Promega Tech Bulliten 009 (Promega; Madison, WI). Restriction enzyme mapping was performed by standard techniques. Gene probes were made by random-primed incorporation of digoxigenin-labelled dUTP and detected after hybridization with a colometric enzyme linked anitibody assay as described in the Genius kit (Boehniger Mannheim Biochemicals; Indianapolis, IN).

A chloride release assay and the detection of metabolites were used to demonstrate dechlorination of 4-chlorobenzoic acid (4-CBA). Chloride released from 4-CBA was measured in chloride free minimal salts buffer using an Orion expandable ion analyzer (model EA-920) equipped with an Orion chloride-selective electrode (Layton, 1992.) Filtered supernatants from cells grown in 4-CBA were analyzed for the presence of 4-CBA and 4-HBA by HPLC. The metabolites were separated on a Supelcosil LC-18 column (Supelco; Bellefonte, PA) and detected with a photodiode array detector (model LC-235; Perkin-Elmer Corp., Groton, Conn) at a wavelength of 255 nm (Layton, 1992).

3. RESULT AND DISCUSSION

3.1 Analysis of Bacterial and NAH gene Distributions in Contaminated Soils.

Manufactured gas plants (MGP) generated gas from coal and oil from the mid-19th through mid-20th century. The major waste products from the gasification process were tars and sludges that mainly consisted by PAH. The purpose of this study was to gain the insight information of biodegradative bacteria in the soils at the genotypic and phenotypic level in response to long term PAH contamination.

3.1.1 Molecular Diagnostics of PAH Biodegradation in Contaminated Soils.

Traditional methods for quantifying specific catabolic bacterial populations underestimate the true population count due to the limitations of the necessary laboratory cultivation methods. Likewise, *in situ* activity is also difficult to assess in the laboratory without altering the sample environment. To circumvent these problems and achieve a true *in situ* bacterial population count and activity measurement, new methods based on molecular biological analysis of bacterial nucleic acids were applied to soils heavily contaminated with PAHs. In addition, a naphthalene-lux reporter system was used to determine bioavailability of naphthalene within these soils.

The summary of the bacterial populations in the contaminated and control soils is shown in Table 4. Among total heterotrophic bacterial populations obtained from test, 2.3 to 61.9% were *nahA* positive as determined by colony hybridization. DNA extracted from seven PAH-contaminated soils and hybridized with the *nahA* gene probe indicated that the naphthalene degradative genes were present in all samples in the range of 0.06 to 0.95 ng/100 ml DNA extract which was calculated to represent 3.2×10^6 to 1.1×10^{10} cells/g soil (assuming one copy of these genes per cell). Messenger RNA transcripts of *nahA* were isolated and quantified from 4

soils. Only one soil tested, soil B, was inducible with salicylate above the *in situ nahA* gene transcript level. Two of the soils, C and G, were already fully induced *in situ*. Naphthalene was bioavailable in soils A, D, E, G, and N as determined by a bioluminescent response from the naphthalene-*lux* reporter system.

Table 4. Comparative bacterial population density among PAH-contaminated and control soils.

Soil	Total bacterial population plate counts	*nahA* population density	
		Colony hybridization[a]	Estimate by DNA extraction[b]
A	$5.6\pm0.8\times10^6$	$1.3\pm0.4\times10^6$	$0.8\text{-}2.8\times10^8$
B	$2.2\pm0.3\times10^6$	$5.9\pm0.8\times10^5$	-[c]
C	$1.2\pm0.2\times10^8$	$3.3\pm0.5\times10^6$	$0.6\text{-}2.4\times10^8$
D	$7.8\pm1.7\times10^6$	$2.1\pm0.5\times10^5$	$0.2\text{-}1.3\times10^7$
E	$1.0\pm0.3\times10^7$	$<2.0\times10^6$	$1.0\text{-}3.6\times10^8$
G	$2.1\pm0.4\times10^8$	$1.3\pm0.3\times10^8$	$0.3\text{-}1.1\times10^{10}$
N	$3.1\pm0.5\times10^8$	$7.1\pm1.8\times10^6$	$1.0\text{-}5.6\times10^7$
Etowah[e]	$1.8\pm0.1\times10^7$	$<3.0\times10^5$	ND[d]
Pamlico[e]	$7.1\pm2.0\times10^6$	$<3.0\times10^5$	ND

[a] Colony forming units/gram soil.
[b] Number of cells/gram soil.
[c] DNA was unable to extract for unknown reason.
[d] ND - not determined.
[e] Control soils.

Analysis of slot blots of the isolated DNA revealed that *nahA* genotype was present in each contaminated soil except soil B. A positive correlation ($r = 0.71$) existed between the naphthalene-degrading bacterial populations determined by direct DNA extraction and colony hybridization (Sanseverino, 1993b). The relationship between the total heterotrophic bacterial population and the naphthalene-degrading bacterial population with average total soil naphthalene is shown in Figure 4. A second-order correlation existed between the naphthalene-

degrading population as determined by colony hybridization (r = 0.95) and by direct soil DNA extraction (r = 0.96; excludes soil E) with total soil naphthalene. Exponential increases in bacterial density occurred when the total soil naphthalene content reached approximately 100 mg/kg soil. Taken together, these data provided information on selection of naphthalene-degrading bacterial population *in situ* and what approaches would be necessary to increase activity.

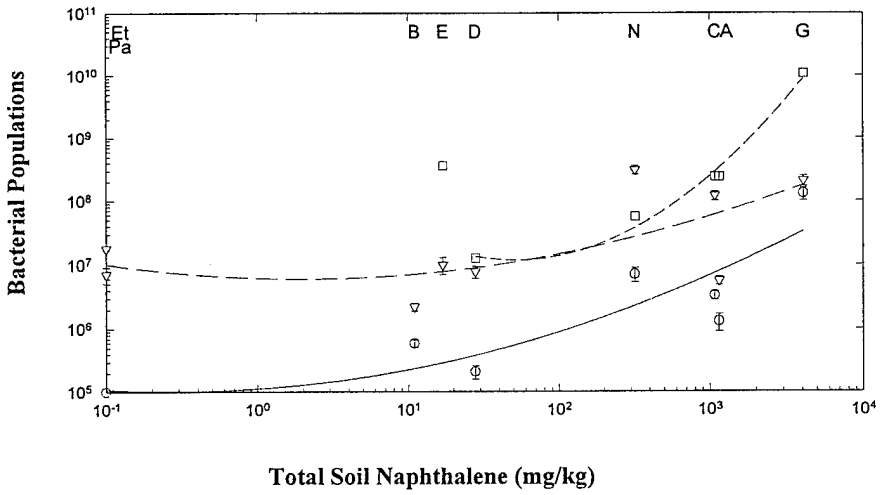

Figure 4. Comparison of the total heterotrophic and *nah*$^+$ naphthalene-degrading bacterial populations as a function of the total soil naphthalene concentration. Naphthalene-degrading bacterial population measurements were by colony hybridization (O; r = 0.950) and direct soil extraction (; r = 0.96; soil E excluded from correlation). Total heterotrophic bacterial population (∇).

3.1.2 Phenotypic Characterization of PAH-Degraders in Contaminated Soils. The results of the phenotypic tests suggested that the PAH degraders in the MGP soils exhibited multiple phenotypes in terms of PAH degradation and showed in Table 5. Among 141 tested

strains, 59 strains were randomly selected strains subjected to the ^{14}C-naphthalene mineralization test. The majority of the naphthalene degraders from the MGP soils were able to degrade 3-ring

Table 5. Multiple phenotypes of PAH-degraders isolated from contaminated soils.

Assay (positive strains/tested strains)	Phenotype	Number of Strains	
Mineralization			
^{14}C-Naphthalene (27/59)	Nah^+Phn^+	6	(22)[a]
	$Nah^+Phn^+Flu^+$	13	(48)
	Nah^+Phn^+ Ant^+	1	(4)
	$Nah^+Phn^+Flu^+Ant^+$	5	(19)
	$Nah^+Phn^+Flu^+Ant^+Pyr^+$	2	(7)
Spray Plate			
Anthracene (13/141)	Ant^+Phn^+	5	(38)
	$Ant^+Phn^+Flu^+$	5	(38)
	$Ant^+Phn^+Flu^+Pyr^+$	3	(23)
Benzo[a]pyrene (0/141)	N/A	0	
Fluorene (35/141)	Flu^+	6	(17)
	Flu^+Phn^+	22	(63)
	$Flu^+Phn^+Ant^+$	4	(11)
	$Flu^+Phn^+Ant^+Pyr^+$	3	(9)
Phenanthrene (83/141)	Phn^+	50	(60)
	Phn^+Flu^+	21	(25)
	Phn^+ Ant^+	4	(5)
	$Phn^+Flu^+Ant^+$	5	(6)
	$Phn^+Flu^+Ant^+Pyr^+$	3	(4)
Pyrene (3/141)	$Pyr^+Phn^+Flu^+Nah^+Ant^+$	3	(100)

a: percentage of the strains that positively scored for the indicated phenotype.
Ant: anthracene; Flu: fluorene; Nah: naphthalene; Phn: phenanthrene; Pyr: pyrene.

PAHs as determined by the spray plate method and the ^{14}C-naphthalene mineralization assays. All naphthalene degraders (27 strains) and anthracene degraders (13 strains) formed clear zones on the phenanthrene spray plates. Three strains (A8AN3, B1PH6, and N1-4PH) produced clear

zones on the pyrene spray plates. The 3 strains could also degrade other PAHs (i.e., fluorene, phenanthrene, anthracene, and naphthalene) as determined by the spray plate method and ^{14}C-naphthalene mineralization assay. No strain, however, produced clear zones on the benzo[a]pyrene spray plates. In the spray plate assays, the time required for the formation of clear zones around colonies was as follows: 1 day for fluorene, 2-3 days for phenanthrene, 3-4 days for anthracene, and 3 weeks for pyrene.

Only 89 out of 141 strains showed PAH degradative ability as determined by PAH spray plate method and ^{14}C-naphthalene mineralization assay, although 141 strains were isolated previously as PAH degraders (King, 1991). The results of the indigo test indicated that 98 % (87/89 strains) of the PAH-degraders showed dioxygenase activity as determined by the formation of indigo. In 32 indigo negative (Ind^-) strains (out of 141), two strains were PAH-degraders, which suggested no or low (under detection limit) dioxygenase activity under the condition used in this study. Among 52 non-PAH degraders, 22 strains scored positive in the indigo test. Not all the strains were subjected to the naphthalene degradation test in this study; therefore, it is possible that some of the strains are naphthalene degraders. The method occasionally produces false results due to cross-feeding of strains on naphthalene vapor plates. In addition, monocyclic aromatic hydrocarbon degraders contain dioxygenases also could convert indole to indigo (Zylstra, 1988; Eaton, 1986; Tan, 1990; Ensley, 1983). It is interesting to note that toluene-4-monooxygenase also has ability to convert indole to indigo (Yen, 1991).

3.1.3 Non-NAH Genotype Strains in Contaminated Soils. Gene probing was used to determine if the isolates contained NAH7-like catabolic genes. NAH7-derived naphthalene dioxygenase gene (*nahA*) was used as a probe in colony hybridization. Of the 141 strains

examined, 96 strains tested positive from the *nahA* gene probing. Among PAH-degraders (89 out of 141 strains), 13 strains (15%) did not hybridize with the *nahA* probe and 4 strains (4%; naphthalene degraders) did not hybridize with any of the NAH7-derived gene probes (*nahA*, *nahG*, and *nahH*; Table 6). In addition, plasmids of these 4 strains also exhibited different *Eco*RI and *Eco*RV restriction patterns from that of NAH7 plasmid. It is possible that these 4 strains may have new *nah* genes which encode different naphthalene degradation pathway from conventional NAH7-encoded pathway. There are considerable amount of non-NAH7 type PAH-degrader reported (Fredrickson, 1991; Foght, 1991; 1988; King, 1991). These 4 strains may serve as new gene probes for monitoring indigenous bacteria population extensively, and for providing insight catabolic activity on *in situ* bioremediation.

Table 6. Characteristics of non-*nahA* probe hybridization strains.

Strain	Indigo Test	*nah* Probe[a] (A,G,H)	14C-Mineralization				Spray Plate		Growth on PIA[b]
			Nah	Phn	Ant	BaP	Flu	Pyr	
A5PH1	+	–	+	+	–	–	–	–	–
A8AN	+	–	+	+	+	+	+	+	–
B1PH2	+	–	+	+	+	–	+	–	–
B10AN1	+	–	+	+	–	–	–	–	–

[a]: NAH7-derived genes probes used for total DNA slot blot hybridization. Plasmid NAH7 DNA from *P. putida* G7 and the total DNA from *P. putida* PB2440 were used as positive and negative controls, respectively.
[b]: Pseudomonad isolation agar.
Ant: anthracene; BaP: benzo[a]pyrene; Flu: fluorene; Nah: naphthalene; Phe: phenanthrene; Pyr: pyrene.

Among these 4 strains, strain A8AN3 was further characterized due to its broader PAH mineralization ability. Strain A8AN3 was determined to be most similar to *Sphingomonas paucimobilis* (Yabuuchi, 1990) based on phenotypic characteristics as well as identification of

sphingolipid and major ubiquinone. A8AN3 contained sphingolipid which is a characteristic component of cellular lipids of the genus *Sphingomonas.* Fatty acid analysis by GC showed that A8AN3 possessed 2-hydroxymyristic acid (2-OH-14:0). Dihydrosphingosine in A8AN3 was identified by GC-MS. Dihydrosphingosine and 2-OH-14:0 are components of sphingolipids in the genus *Sphingomonas*. Isoprenoid quinone analysis by HPLC showed that ubiquinone 10 (Q10), major quinone in the genus *Sphingomonas*, was the major type of ubiquinone in A8AN3. A8AN3 had a polar monotrichous flagellum as shown by transmission electron microscopy (Univ. of Tennessee - Knoxville, Dept. of Zoology, EM Facility). The yellow pigment of A8AN3 had an absorbance spectrum that matched nostoxanthin (Holmes, 1977), the pigment of *S. paucimobilis* ATCC 29837.

3.2 Application of Bioluminescent Reporters in Monitoring Environmental Pollutants. The *nah-lux* bioreporter strains, HK44 and 5RL, were used in this study to monitor bioavailability of environmental pollutants, such as naphthalene, salicylate; and JP-4 jet fuel.. Furthermore, a whole-cell biosensor was developed for a continuos, on-line monitoring of microbial catabolic activities in waste stream.

3.2.1 *nah-lux* Reporter - Biosensing for Pollutant Availability for Biodegradation in Environmental Systems. A bioassay was developed and standardized for rapid, quantitative and specific assessment of naphthalene and salicylate. The bioluminescent catabolic reporter strain *Pseudomonas fluorescens* HK44, which carries a transcriptional *nahG-luxCDABE* fusion for naphthalene and salicylate metabolism, was used. A good linear correlation between bioluminescence and naphthalene or salicylate aqueous phase concentration was found over a range of 1 to 2 orders of magnitude, using exponentially growing reporter cultures (OD_{546}=0.35).

A significant bioluminescence response could be detected for naphthalene concentration of 45 ppb. Studies conduct under defined conditions with extracts and slurries of experimentally contaminated sterile soil and identical uncontaminated soil controls demonstrate that this method can be used for specific and quantitative estimations of target pollutant presence and bioavailability in soil extracts and for specific and qualitative estimations of naphthalene in soil slurries. In addition, the carbon starved resting cultures can cause significant bioluminescence increase upon exposure to readily metabolizable substrates such as glucose, peptone and yeast extract, thereby reducing the specificity of the response. Studies conduct in aqueous extracts of environmental soil/sediment samples contaminated with petroleum hydrocarbons have demonstrated increasing bioluminescence response as compare to uncontaminated control samples.

The correlation between bioluminescence and biodegradation is examined in dual carbon substrate system, which containing 1 g/l glucose and 3 or 12 mg/l salicylate, using *Pseudomonas fluorescens* HK44 in a define mineral salts medium. Preliminary data show that a positive correlation exists between the integrated bioluminescence signal and the corresponding amounts of degraded salicylate.

Earlier studies demonstrated that bioluminescent reporter bacteria can be immobilized in calcium alginate which provides a translucent matrix. Exposure of *Pseudomonas fluorescens* HK44 immobilized in alginate brads to different naphthalene and salicylate concentrations result in transient bioluminescence responses. Good linear correlations were found between peak bioluminescence levels and target pollutant concentrations.

3.2.2 Environmental Application of *nah-lux* and *tod-lux* Bioreporters. A bioluminescent reporter bacterium *Pseudomonas fluorescens* HK44 for naphthalene and salicylate catabolism was used for analysis of naphthalene and bioavailability in aqueous extracts of soils contaminated with petroleum hydrocarbons. For each experimental set which involved two different soils, one presumably uncontaminated soil A and a heavily contaminated soil B, a series of naphthalene concentrations were included in order to prepare bioluminescence versus naphthalene standard curve. For all the samples investigated, the final biomass concentrations were identical and remained unaffected by the contaminants. A very reproducible bioluminescence response was obtained from contaminated extracts as compared to extracts from an uncontaminated control soil; however, quantitative estimates of the amount of naphthalene present in the mixture were not accurate.

On studies conducted with JP-4, jet fuel experimentally contaminated samples a linear correlation between the relative amount of pollutant and the magnitude of the bioluminescence response was observed. In Figure 5 the bioluminescence response of *P. fluorescens* HK44 to different amounts of JP-4 jet fuel is shown. The dilution factors used referenced to an aqueous solution, saturated with JP-4. A good linearity was found for the dilution investigated, which included 12 samples with a range from 0.03 to 0.5 and a control with water. The regression line was described by the equation: $y = 104.83x + 1.2656$ with $r^2 = 0.967$. For all the samples investigated, the final biomass concentrations were identical and remained unaffected by the amount of JP-4.

In Figure 6 there is also significant response to the aqueous solution containing JP-4 by *P. putida* B2. This figure also shown that maximum toluene gene expression occurs at 10 mg per

liter toluene. This provides direct evidence that in the case of the toluene dioxygenase cooxidation of TCE 10 mg per liter toluene causes maximum induction of the system.

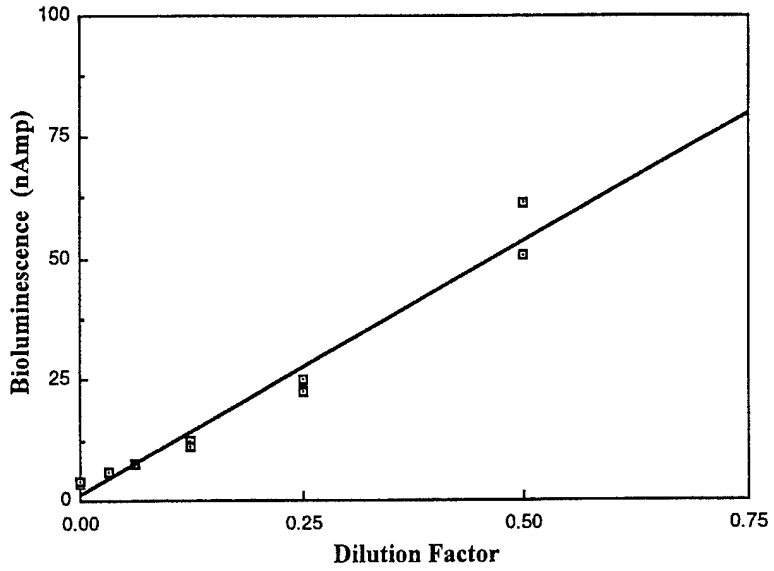

Figure 5. Relationship between the amount of JP-4 jet fuel and the bioluminescence response of growing cultures of P. fluorescens HK44 after 1 hour incubation. The amount of JP-4 is expressed as a factor of an aqueous solution, saturated with JP-4.

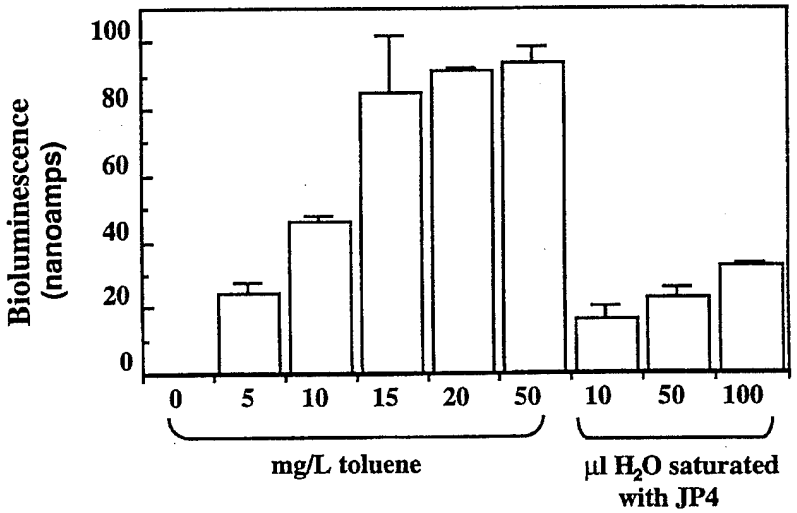

Figure 6. Specific bioluminecence of *Pseudomonas putida* B2 after exposure to different concentrations of toluene and water saturated with JP-4 fuel.

It was interesting to note that 64 mg/l toluene, 22 mg/l *p*-xylene and 980 mg/l acetone caused a significant bioluminescence increase as compare to a control with water. In addition, the mixtures of the same concentrations of either acetone and toluene or acetone and *p*-xylene the responses were exactly additive but not for the mixture of toluene and *p*-xylene. The solvent effects were dependent on the physiological status of the reporter culture and were present in growing, but not in resting reporter cultures.

In order to determine the relationship between bioluminescence response and pollutants, a mRNA extraction was performed on 4 ml culture in the test vials. The analysis of *nah-lux* mRNA in growing cultures revealed that even though toluene had a strong effect on the bioluminescence response in growing reporter cultures after exposure to the solvent, but it did not increase *nahG-luxCDABE* gene expression. In contrast, *nah-lux* mRNA levels significantly increased after exposure to naphthalene or JP-4 jet fuel. It is proposed that the increase in bioluminescence after exposure to solvents was due to changed fatty acid synthesis patterns affecting the aldehyde supply for the bioluminescence reaction.

3.2.3 Bioluminescent Biosensor for On-Line Pollutant Monitoring. An optical whole cell biosensor based on the bioluminescent catabolic reporter bacterium, *Pseudomonas fluorescens* HK44, was developed for continuous, rapid on-line monitoring of naphthalene and salicylate bioavailability and microbial catabolic activity in waste stream, i.e. "dirty" samples. The reporter strain, *P. fluorescens* HK44 was capable to degrade naphthalene and salicylate.

Under defined conditions, a rapid increase in bioluminescence was detected after exposure to naphthalene and salicylate. The magnitude of the response and the response time were concentration dependent. The response time was defined as the time interval between

exposure to the pollutant and the time when the bioluminescence response exceeded the sum of three standard deviations of the average baseline value prior to induction. It was interesting to note that at lower inducing substrate concentrations of 0.5 mg/l salicylate and 1.55 mg/l naphthalene, the response times of 24 minutes were significantly longer than at high concentrations. Under repetitive perturbation conditions, good reproducibilities of the signal magnitude and response time were found for both substrates, naphthalene and salicylate. The specificity of the biosensor was determined by examining the response of different carbon sources in the waste stream and compared to the response to naphthalene. The bioluminescent responses were insignificant (less than 2 fold) and response times were significantly longer than those observed for naphthalene (1.55 and 15.5 mg/l) when glucose (1.0 g/l) and YEPG medium (1.0 g/l) were used as substrate. Exposure to toluene resulted in no significant bioluminescence signal. The environmental application of the biosensor was tested using real, complex pollutant mixtures containing naphthalene. Naphthalene concentration was detected at 0.55 mg/l in the effluent of the biosensor. A positive bioluminescence response was also observed after exposure to the aqueous leachate from a manufactured gas plant (MGP) soil. The concentration of naphthalene was estimated at 0.6 mg/l in the effluent of the soil column.

3.2.4 Determination of Bioluminescent Response to PAH Priority Pollutants and Metabolites. Two bioluminescent reporter strains *P. fluorescens* HK44 (*nah*$^+$, *sal*$^+$) and 5RL (*nah*$^+$, *sal*$^-$) were used in this study. The results of one-hour exposure assays with *P. fluorescens* HK44 using saturated solutions (without crystals) shown that salicylate, naphthalene and acenaphthylene producing significant light increase as compared to the control without inducing substrates. All other compounds, acenaphthene, anthracene, benzanthracene, chrysene,

dibenzanthracene, fluorene, fluoranthene, methyl-anthracene and phenanthrene, did not increase the catabolic *nahG* expression over the background control. In prolonged exposure assays (5.5 hr) and the use of aqueous solutions containing crystals resulted in a significant induction of catabolic gene expression with several compounds which tested negatively in strain HK44, such as anthracene, benzo[a]pyrene, fluorene and phenanthrene. A similar bioluminescence response patterns are observed in the strain 5RL after 3.6 hour exposure to the PAHs. The above results suggested that the naphthalene degradation pathway in *P. fluorescens* HK44 and 5RL was not only induced after exposure to naphthalene but might also be induced upon exposure to acenaphthene, acenaphthylene, fluorene, phenanthrene ,anthracene and benzo[a]pyrene.

In addition, it has been demonstrated with 5RL that the intermediate of anthracene degradation, 2-hydroxy-3-naphthoic acid, can act as an inducer of the naphthalene degradation pathway. But, the similar light induction response is not observed with 1-hydroxy-2-naphthoic acid, the intermediate in phenanthrene degradation.

3.3 Maintenance of Wild type (pKA1) and Bioluminescent Reporter Plasmids (pUTK9 and pUTK21) Under Non-Selective Conditions in Continuous Culture. The wild type naphthalene degradative plasmid, pKA1, was maintained in 100% of the *P. fluorescens* 5R culture on minimal glucose media. The plasmid was maintained for 94 generations at a dilution rate of 0.171 hr^{-1} and an additional 27 generations at a dilution rate of 0.257 hr^{-1} (Figure 7). All colonies on the YEPG plates were indigo positive, a presumptive test for dioxygenase activity (*nahA* in this case).

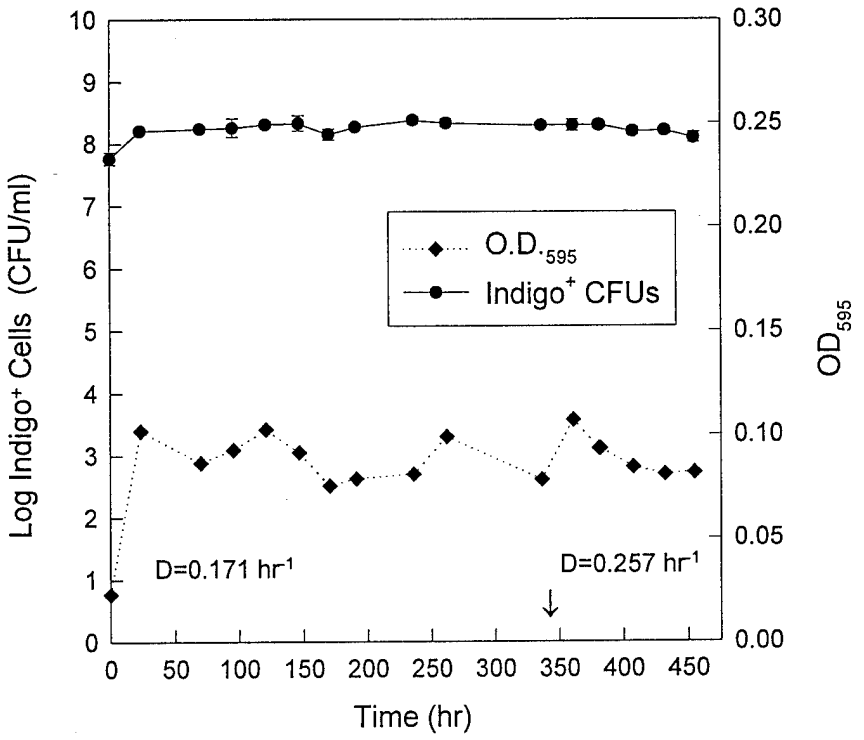

Figure 7. Maintenance of pKA1 in *P. fluorescens* 5R growing in continuous culture on 100 ppm glucose in minimal media.

The maintenance of the naphthalene biodegradation reporter plasmid pUTK21 in glucose minimal media is shown in Figure 8. The plates counts from tetracycline selective media (cells retaining pUTK21) closely follows the *lux*AB probed colony blots taken from the plates without tetracycline (total CFUs). These results show that 40% of the culture exists without pUTK21 as shown in Figure 9. The initial fraction of the culture retaining pUTK21 is shown as 90% but could be attributed to plating error since the inoculum was grown under tetracycline selection. The ratio of the culture retaining pUTK21 declined to 60% over 98 generations at a dilution rate of 0.171 hr^{-1}.

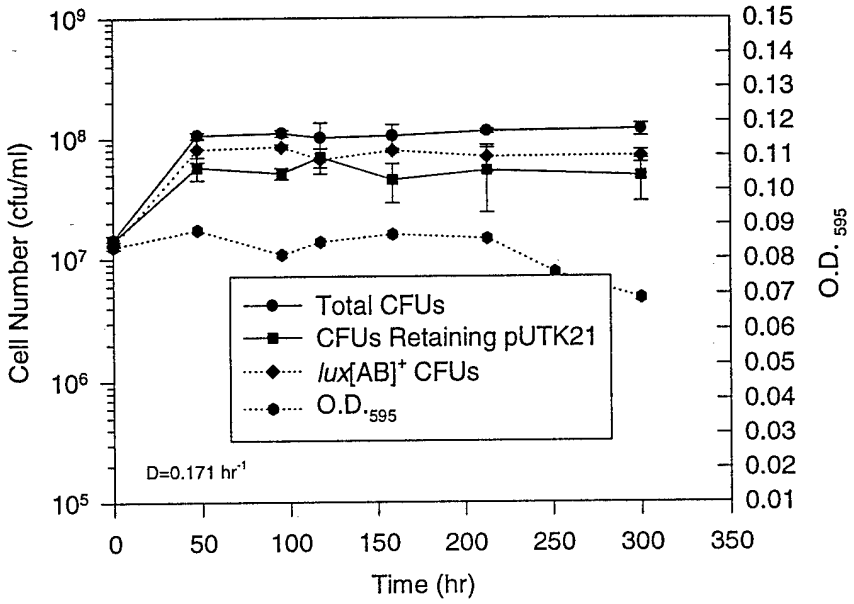

Figure 8. Maintenance of pUTK21 in *P. fluorescens* 5RL growing in continuous culture on 100 ppm glucose in minimal media.

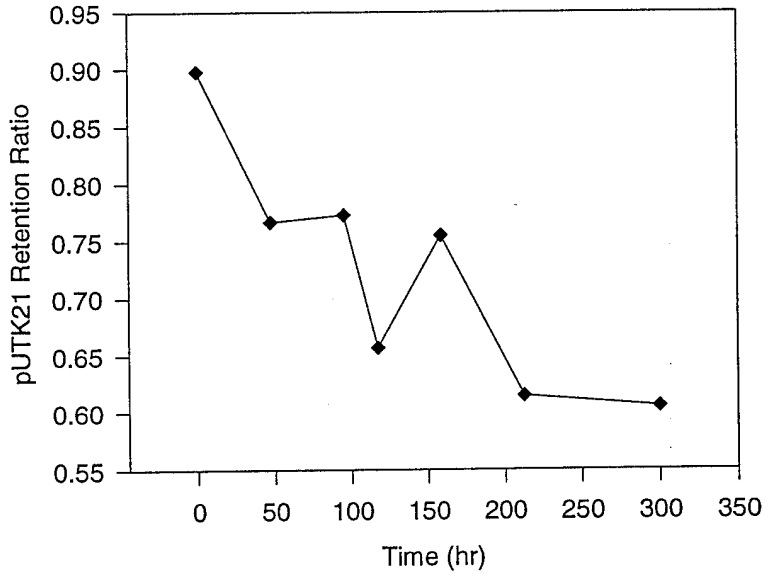

Figure 9. The plasmid retention ratio of pUTK21 in *P. fluorescens* 5RL (total CFUs/CFUs retaining pUTK21) is shown (♦).

The maintenance of pUTK21 in *P. fluorescens* HK44 is shown in Figure 10. The data show that the portion of the population retaining the reporter plasmid declined to 1.58 X 10^4 cfu/ml after 39 generations at a dilution rate of 0.086 hr^{-1}. This decrease coupled with a 1.6 X 10^8 cfu/ml increase in total cells resulted in 99% loss of pUTK21 in the HK44 culture (Figure 11). The plasmid bearing portion of the culture was maintained at the same level for another 23 generations. An increase in the dilution rate to 0.171 hr^{-1} had no significant effect in plasmid maintenance for an additional 13 generations. Plasmid preparations derived from random culture isolates showed that the Tn4431 transposon in pUTK21 remained stable. The plasmid preparations from individual strains were *Bam*HI digested, southern blotted and probed with a *lux*AB specific DNA probe. All isolates retaining pUTK21 also retained the *lux* insertion consistent with the expected 7.5 kb fragment compared to standard pUTK21 DNA. All isolates retaining pUTK21 were tetracycline resistant whereas those isolates that lost pUTK21 were tetracycline resistant. This indicates that the transposon did not insert in a chromosomal site. Although these blots were not probed for naphthalene degradative genes, there did not appear to be any deletions or rearrangements in any of the isolates since all isolates maintained the same DNA fragment pattern. Additionally, all isolates that lost pUTK21 had the plasmids that are present in the parent 18H strain and this precludes the possibility that the isolates were merely contaminants. The plasmid retention ratios for the wild type plasmid pKA1 and the engineered plasmid pUTK21 are summarized in Figure 12.

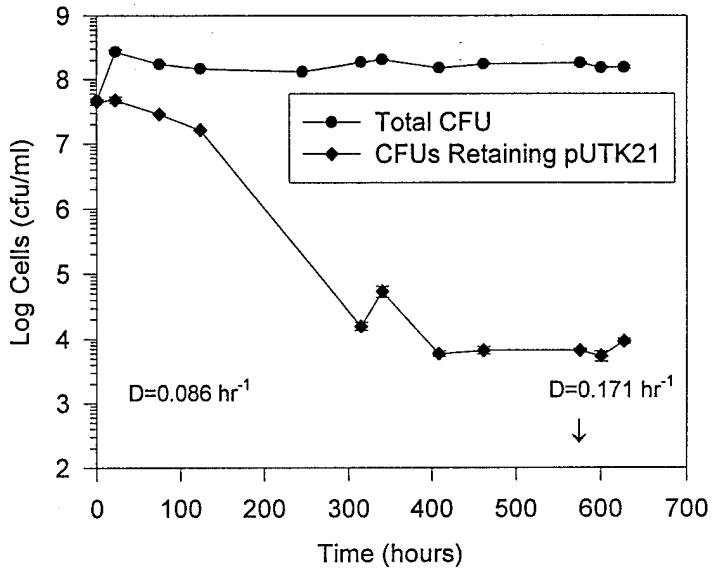

Figure 10. Maintenance of pUTK21 in *P. fluorescens* HK44 growing in continuous culture on 100 ppm glucose in minimal media. Total cells (•), CFUs retaining pUTK21 (♦) are shown.

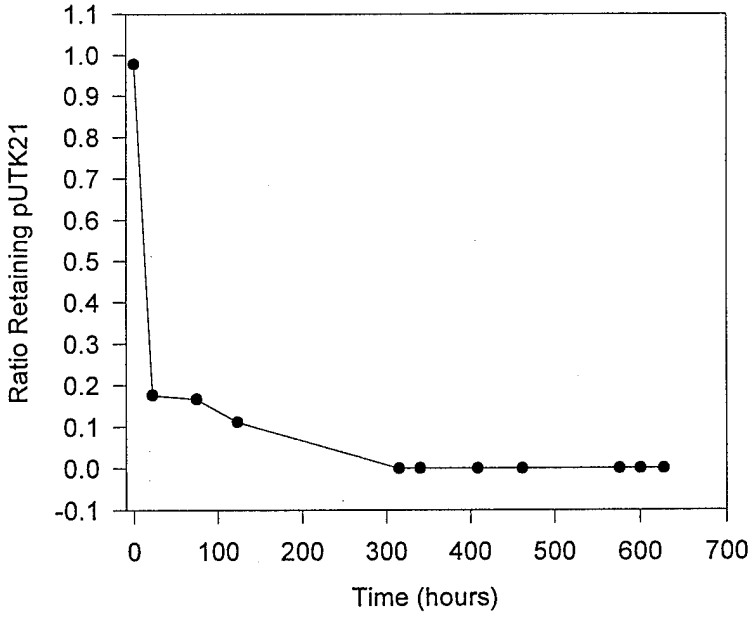

Figure 11. The plasmid retention ratio of pUTK21 in *P. fluorescens* HK44 (total CFUs/CFUs retaining pUTK21) is shown (•).

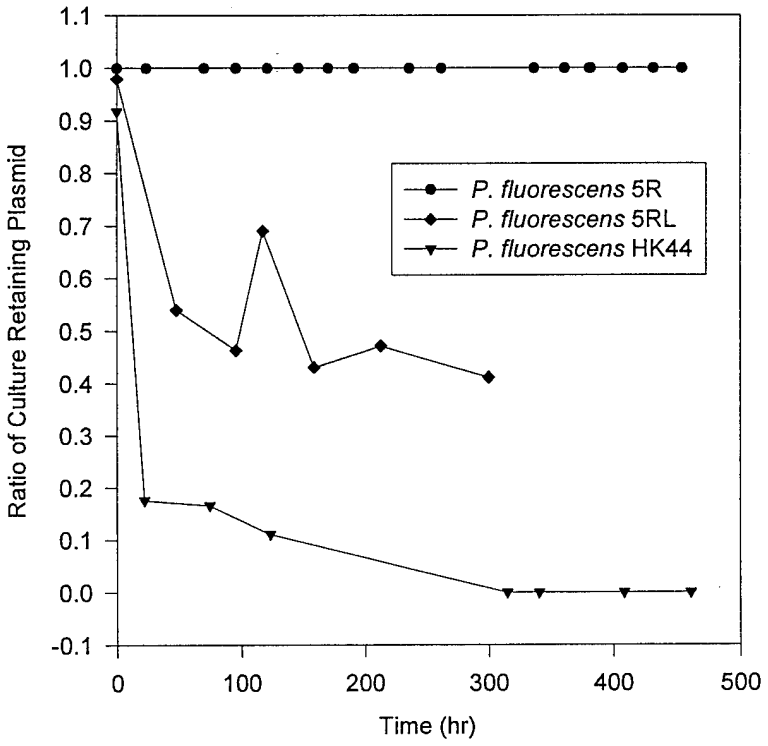

Figure 12. The plasmid retention ratios of pKA1 in *P. fluorescens* 5R (•) and pUTK21 in *P. fluorescens* 5RL (♦) and *P. fluorescens* HK44 (▼) are shown.

The maintenance of the reporter plasmid pUTK9 in *P. putida* RB1351 is shown in Figure 13. This data was derived from a naphthalene competition experiment and does not represent data from a RB1351 culture grown on glucose minimal media. Since naphthalene minimal media does not select for pUTK9 (contrast to pUTK21), there was no selection for pUTK9 and this experiment served the purposes of demonstrating maintenance in a non-selective medium. The data show that the reporter plasmid pUTK9 was maintained in 67-95% of the *P. putida* RB1351 population (Figure 14). The population of *P. putida* RB1351 lacking pUTK9 fluctuates, but there is no identifiable trend in plasmid loss. This lack of a trend is due to the difficulty in monitoring this population at such low numbers.

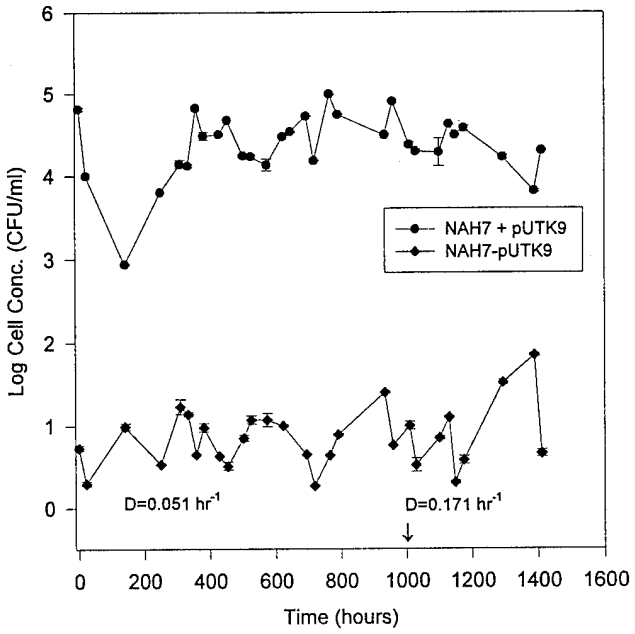

Figure 13. Maintenance of pUTK9 in a chemostat culture containing *P. putida* RB1351 growing on saturating levels of naphthalene in minimal media. The cells retaining pUTK9 (•) and the cells that have lost pUTK9 (♦) are shown.

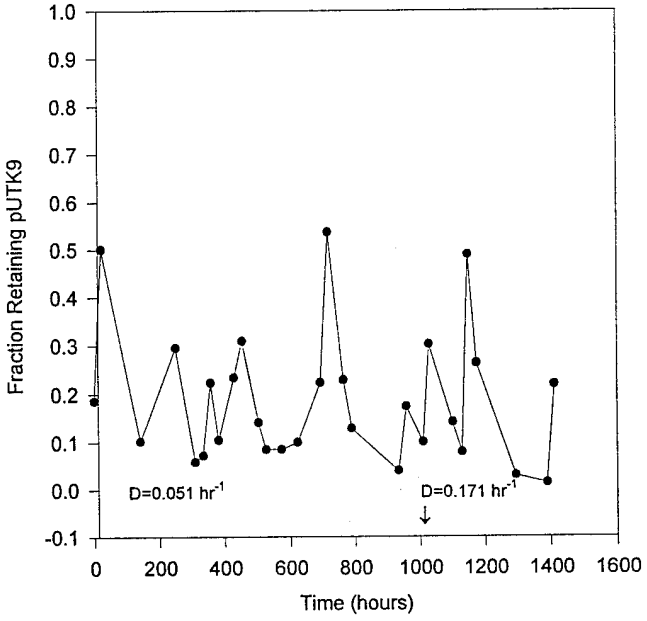

Figure 14. The plasmid retention ratio of pUTK9 in *P. putida* RB1351 (total CFUs/CFUs retaining pUTK9) is shown (•).

3.3.1 Competitive Maintenance of Engineered and Wild Type Strains. Population maintenance of the wild type degrading strain *P. fluorescens* 5R and two engineered reporter strains (*P. putida* RB1351 and *P. fluorescens* 5RL) growing on saturating levels of naphthalene minimal medium is shown in Figure 15. The data show that the wild type strain was maintained at a level 3-5 orders of magnitude higher than either engineered strain. The wild type strain *P. fluorescens* 5R, *P. fluorescens* 5RL, and *P. putida* RB1351 were maintained at 10^8, 1.3 x 10^3 and 5 x 10^4 cfu/ml respectively at a dilution rate of 0.017 hr^{-1} for 25 generations. At a dilution rate of 0.17 hr^{-1}, the *P. fluorescens* 5R and *P. putida* RB1351 were maintained as before for an additional 99 generations, but *P. fluorescens* 5RL was nearly washed out. The results do show that *P. putida* RB1351 was maintained at 10^4-10^5 cfu/ml even at higher dilution rates. *P. fluorescens* 5RL was nearly washed out at higher dilution rates, possibly due to the inability of the strain to mineralize naphthalene completely (accumulates salicylate).

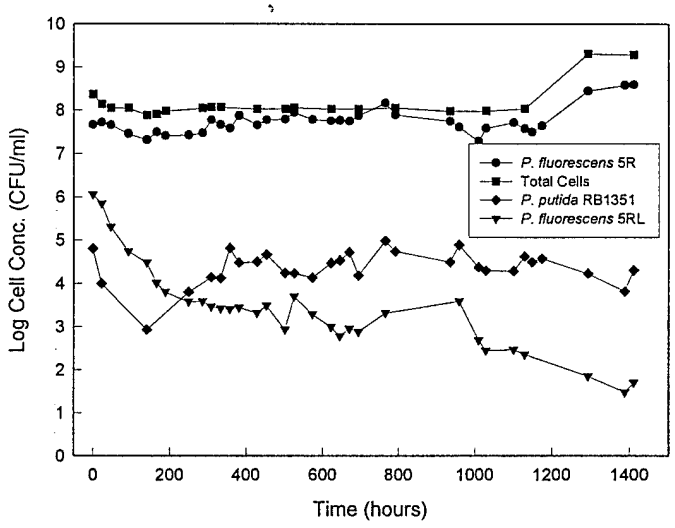

Figure 15. Population maintenance of a wild type PAH degrading strain *P. fluorescens* 5R (•), and two engineered reporter strains; *P. putida* RB1351 (♦), and *P. fluorescens* 5RL (▼) growing on saturating levels of naphthalene in minimal media.

The sharp increase in total cell number and in the population level of *P. fluorescens* 5R is unexplainable. No naphthalene or metabolites were detected in the culture effluent (data not shown). The fact that no salicylate was detected in the culture effluent (data not shown) indicates that *P. fluorescens* 5RL provided an additional substrate for growth to *P. fluorescens* 5R and *P. putida* RB1351, thus providing a competitive advantage to those strains.

A similar mixed culture was composed of *P. putida* RB1351, *P. fluorescens* 5Rrif, and *P. fluorescens* HK44. Because *P. fluorescens* HK44 has a complete salicylate degradation pathway, it was hypothesized that this strain could compete with the other strains for naphthalene and be maintained in continuous culture. The maintenance of the respective populations is shown in Figure 16.

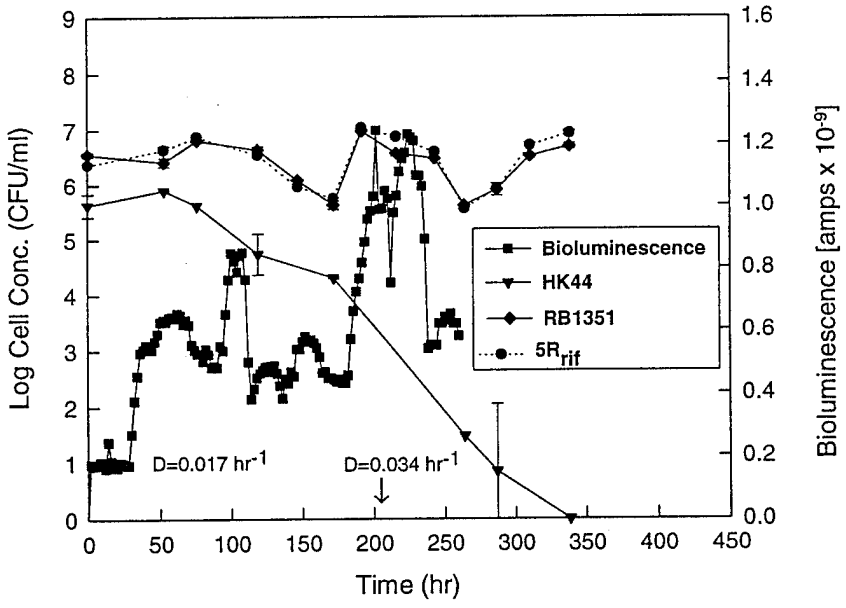

Figure 16. Population maintenance of a wild type PAH degrading strain *P. fluorescens* 5Rrif (•) and two engineered reporter strains *P. putida* RB1351 (♦), and *P. fluorescens* HK44 (▼), growing on saturating levels of naphthalene in minimal media. Bioluminescence is indicated as (■).

The wild type strain *P. fluorescens* 5Rrif and *P. putida* RB1351 were maintained at 1.2-8.2 x 10^6 cfu/ml for 5 generations at a dilution rate of 0.017 hr^{-1}. The population of *P. fluorescens* HK44 dropped from 5 x 10^5 to 4 x 10^5 cfu/ml. At a dilution rate of 0.034 hr^{-1}, *P. flourescens* 5Rrif and *P. putida* RB1351 increased to 9 x 10^6 cfu/ml, gradually decreased to 4 x 10^5 cfu/ml, then again increased to 5 x 10^6 cfu/ml over 8.2 generations. *P. fluorescens* HK44 gradually decreased until no CFUs could be detected. This eventual washout of *P. fluorescens* HK44 may have been due to the 10-fold lower inoculum of this strain even though there was an attempt to inoculate all strains at the same titer. This situation would provide a competitive advantage to the strains present in higher numbers.

The bioluminescent response of the mixed culture was dynamic in character. Upon inoculation of the respective strains, bioluminescence increased to 0.2 x 10^{-9} amps and remained constant until the continuous addition of medium after 24 hr. At this time, the bioluminescence increased to 0.63 x 10^{-9} amps. The response fluctuated over the next 120 days. Upon increasing the dilution rate from 0.017 hr^{-1} to 0.034 hr^{-1}, the bioluminescence increased 2.5 fold, fluctuated, then decreased to 0.6 x 10^{-9} amps. This data demonstrates that the bioluminescent response is sensitive to perturbations such as a change in dilution rate. A steady state was regained, indicating that the bioluminescent response is probably not related to growth rate.

Because *P. fluorescens* HK44 was not maintained in the previous experiment, this strain was continuously cultured alone with naphthalene as a sole source of carbon. The experiment was set up to test the hypothesis that *P. fluorescens* HK44 was not maintained in mixed continuous culture due to the loss of pUTK21. Although naphthalene serves as selective pressure for pUTK21, it is conceivable that the plasmid is gradually lost over time and the host

strain washes out of the culture. The culture was monitored using selective and non-selective media. If a large portion of the population were losing pUTK21, then large differences in plate counts on the respective media would be expected. There were no such differences (data not shown). The population maintenance of *P. fluorescens* HK44 is shown in Figure 17. The data show that the population started at 1.2×10^6 cfu/ml. This level increased to 1×10^8 - 7×10^7 cfu/ml and was maintained for 9 generations at a dilution rate of 0.026 hr^{-1}. The culture was maintained for another 17 generations at a dilution rate of 0.06 hr^{-1}. Upon addition of *P. fluorescens* 5Rrif, the population of *P. fluorescens* HK44 decreased to 5×10^7 cfu/ml but was maintained for another 21 generations at a dilution rate of 0.06 hr^{-1}.

As determined by probing colony blots taken from culture samples with *lux*AB specific DNA, the maintenance of pUTK21 is shown in Figure 18. The results agree with the viable

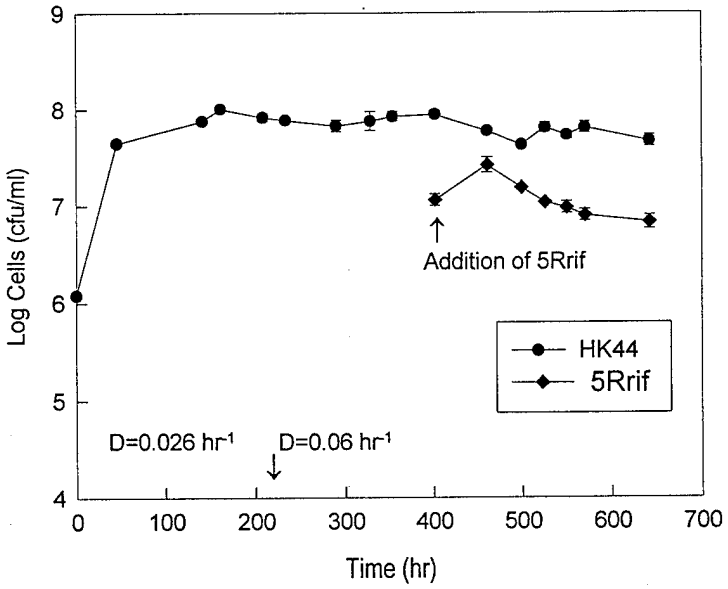

Figure 17. Population maintenance of *P. fluorescens* HK44 (•) growing on saturating levels of naphthalene in minimal media in continuous culture. The wild type PAH degrading strain, *P. fluorescens* 5Rrif (♦), was inoculated into the culture after 17 days of cultivation.

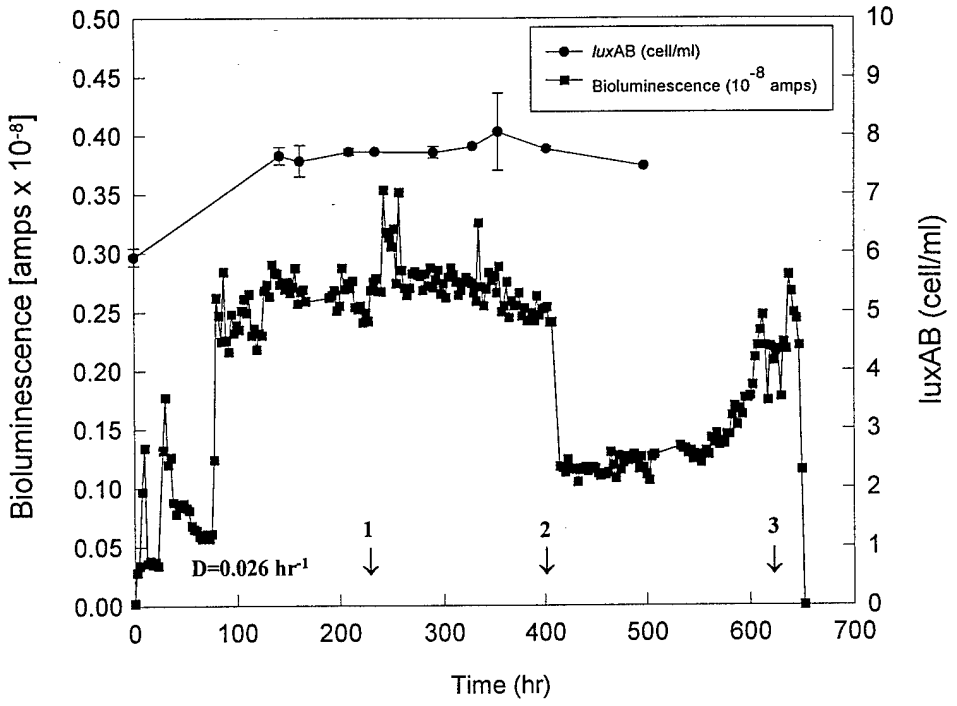

Figure 18. Maintenance of pUTK21 (l) in *P. fluorescens* HK44 growing in continuous culture on saturating levels of naphthalene in minimal media. The bioluminescent response is indicated (■). The system was perturbed in the following ways: (•) The dilution rate was increased to 0.06 hr^{-1}; (2) The strain *P. fluorescens* 5Rrif was inoculated into the system; (3) The culture was poisoned with the detergent Quatsyl 256.

count data shown in Figure 17. These data indicate that the reporter plasmid pUTK21 is not lost during continuous culture on naphthalene. The *lux* genotype was stable maintained for 9 generations at a dilution rate of 0.026 hr^{-1}. Upon increasing the dilution rate to 0.06 hr^{-1}, the *lux* genotype continued to be maintained for another 17 generations. When the wild type PAH degrading strain *P. fluorescens* 5Rrif was added to the culture, the *lux* genotype continued to be maintained for another 21 generations.

The bioluminescent response of the culture is also shown in Figure 18. Over the first 3 generations, the bioluminescence was dynamic but reached a steady-state at 0.27 x 10^{-8} amps.

These data agree with the viable counts shown in Figure 17 with respect to cell number and bioluminescent output. The bioluminescence becomes steady about the same time the viable count of *P. fluorescens* HK44 becomes steady. The bioluminescence remains steady for 9 generations at a dilution rate of 0.026 hr^{-1}.

Upon increasing the dilution rate to 0.06 hr^{-1}, the bioluminescent response shows a brief fluctuation and then attains its former level for another 6.5 generations. This fluctuation was much smaller in magnitude than the one in Figure 16. The dilution rates were different and it is impossible to judge whether such a perturbation is more pronounced in a mixed culture than in a pure culture. Upon addition of *P. fluorescens* 5Rrif to the culture, the bioluminescence dropped two fold. The bioluminescence regained its former level after an additional 21 generations. It seems that the sudden burden of competition has a pronounced effect on the bioluminescent response in this situation. At the conclusion of the experiment, the culture was poisoned with the detergent Quatsyl^R 256. The bioluminescence dropped to 0 x 10^{-8} amps. This demonstrates that the response was indeed due to light from the culture and not due to light leakage or dark current.

3.3.2 Persistence of Engineered Reporter Strain in Mixed Culture Degrading Mixed Contaminants. The *lux* reporter strain *P. putida* RB1351 could successfully compete with a wild type undefined assemblage. Population maintenance of *P. putida* RB1351 in a mixed culture containing a community derived from a PAH contaminated soil is shown in Figure 19. The data show that after 11.5 generations at a dilution rate of 0.171 hr^{-1}, the RB1351 reporter strain was maintained at a level of 2.4-2.6 x 10^6 cfu/ml. The wild type assemblage was maintained at 2.3 x 10^6-8.5 x 10^6 cfu/ml. No naphthalene was detected after day one, and phenanthrene could not be

detected after day six. The abrupt rise in the level of the wild type assemblage correlates with the utilization of phenanthrene. *P. putida* RB1351 is unable to utilize phenanthrene, thus the wild

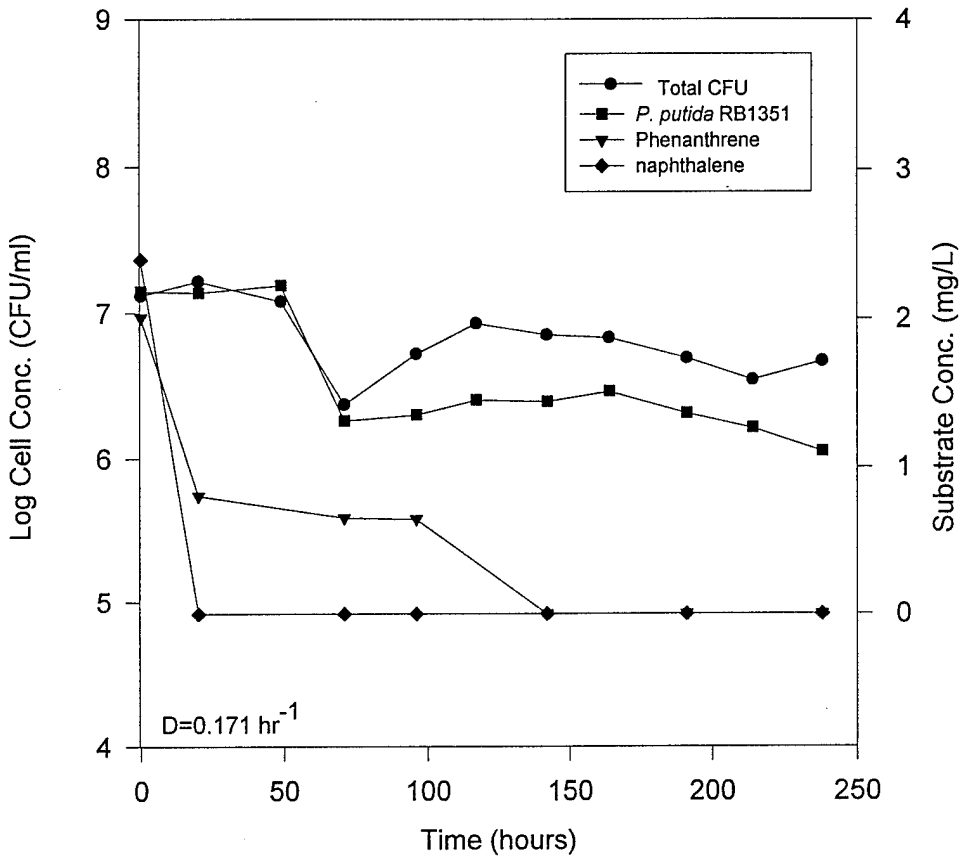

Figure 19. Maintenance of an engineered *lux* reporter strain *P. putida* RB1351 (■) in a mixed culture containing a community derived from a PAH contaminated soil. The total CFUs (•) are indicated. Naphthalene (♦) and phenanthrene (▼) were supplied at saturating concentrations in minimal media supplemented with a soil extract (DOC = 10 mg/ml).

type assemblage may have gained a competitive advantage from an additional carbon source.

The *lux* strain *P. putida* 1351 was capable of maintaining a detectable output of bioluminescent light for long periods of time. Bioluminescence from this strain in a mixed culture (same as Figure 19) is shown in Figure 20. The results indicate that bioluminescence was

maintained at 1.4-2.3 x 10^{-8} amps. The gap in the data was due to a computer malfunction and subsequent loss of data.

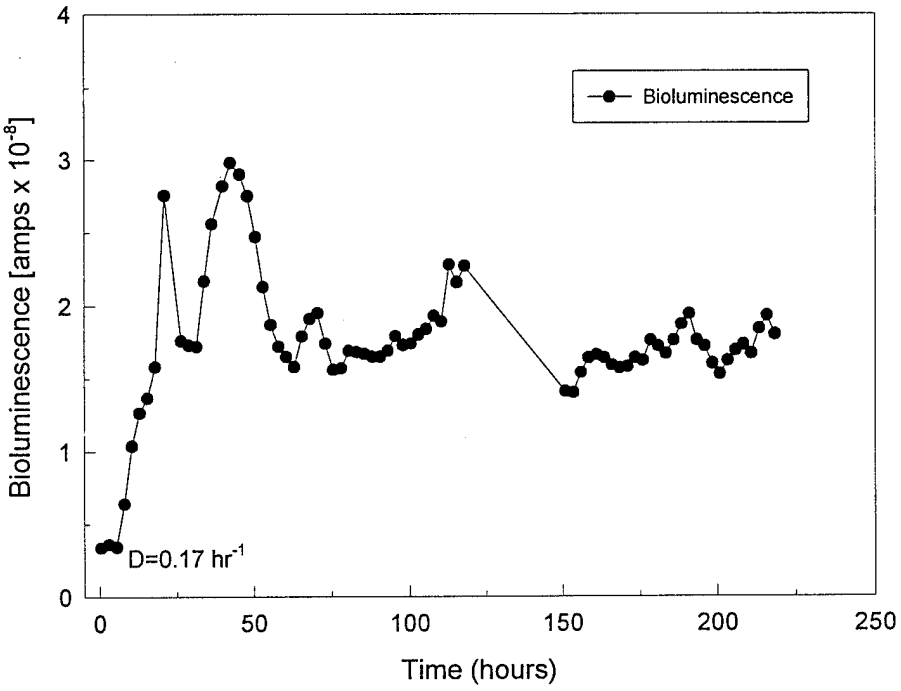

Figure 20. Bioluminescence from a lux reporter strain *P. putida* RB1351 in a mixed culture containing a community derived from a PAH contaminated soil. Media conditions were the same as in Figure 19.

3.4 Construction of *tod-lux* Reporter and Co-Oxidation of TCE. The reporter plasmid pUTK 30 was generated by cloning the *tod* promoter in front the lux genes of pUCD615 (Rogowsky, 1987). This was accomplished by directionally cloning a 2.75 kb *Eco*RI-*Xba*I fragment from pDTG514 (Menn, 1991; Figure 21). Plasmid minipreps were cleaved with *Bam*HI to confirm insertion of the *tod* fragment. The resultant reporter plasmid pUTK 30 was mated into *P. putida* F1 and exconjugants were selected for light production on LB plates

containing kanamycin (50 μg /ml) and toluene vapor. Of five of the exconjugants were selected for further study, strain B2 was arbitrarily picked to be used in the remaining experiments.

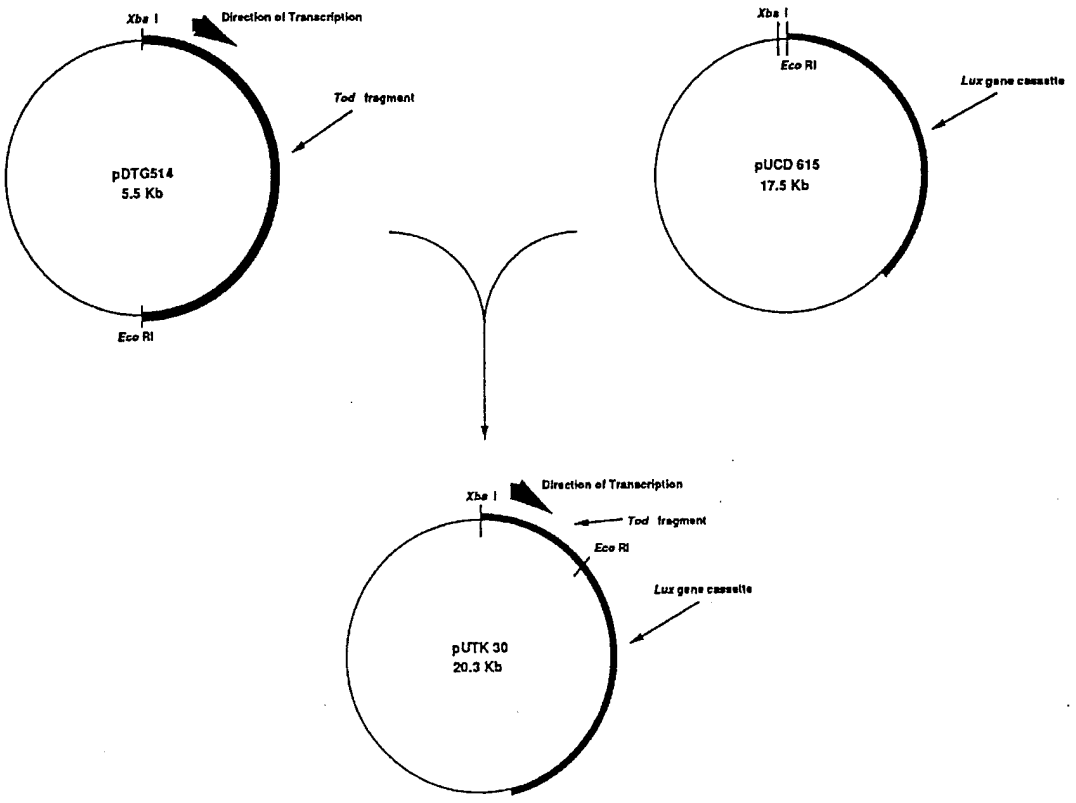

Figure 21. Schematic diagram of the construction of the *tod-lux* reporter plasmid pUTK30.

The co-metabolism of TCE by *tod-lux* reporter was conducted in a differential volume reactor (DVR). The DVR system inoculated with B2 immobilized in alginate beads was used to determine the light response of the strain when induced by toluene. The inlet concentration of toluene was altered by using square-wave perturbations having 20 hr cycles. During feed portions of the cycle 10 mg/L toluene was introduced into the inlet of the reactor. Preliminary experimental results exhibited a very rapid and seemingly instantaneous bioluminescent response

within the differential volume reactor upon the introduction of toluene. Figure 22 shown both the light response of the reporter strain in the reactor and the step changes made with the inlet concentration of the inducer indicated a direct response of bioluminescence of B2 with respect to toluene present in the system. During the cycle, light emission increased by 16.3 ± 1.2 nAmp/hr. The toluene effluent concentration approached zero after the toluene feed was stopped, and the light response in the reactor decreased at a rate of 3.4 ± 0.8 nAmp/hr.

In Figure 22 also shown TCE was degraded by the toluene dioxygenase in F1 after the *tod-lux* reporter induced by toluene. A direct correlation between light emission and TCE degradation was observed. The maximum light response was 43.4 ± 6.8 nAmps. The system degraded approximately 20% of the TCE and 50% of the toluene. This gave a steady-state TCE effluent concentration when toluene was being introduced into the system of 16.5 ± 0.2 mg/L,

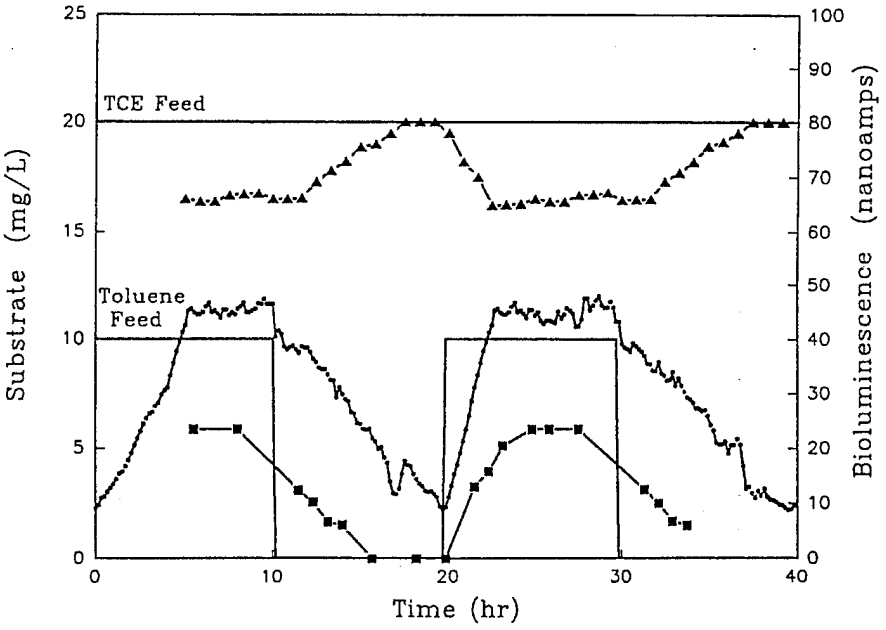

Figure 22. Bioluminescent response and cometabolism of TCE by *P. putida* B2 to square wave perturbations of 10 mg/ml toluene in 20 hour cycles. Symbols used in the Figure describing as follow: ● bioluminescence; ▲ TCE effluent; and ■ toluene effluent.

and an effluent toluene concentration of 5.8 ± 0.1 mg/L, which represents a ratio of 1.7 μmol toluene degraded/μmol TCE degraded. Approximately 50% of the toluene was degraded by the strain B2 in the DVR (Figure 22). This experiment was performed to ascertain the effect of the *lux*-reporter plasmid in the degradative capacity of *P. putida* F1.

The system described here showed a direct correlation of TCE biotransformation to the bioluminescent response of the strain *P. putida* B2. The results of the square wave perturbations were similar to the response obtained by King *et al* (1990) with the naphthalene system. These results show the utility of such a strain's use in the monitoring of TCE biotransformation. The strain could be used to monitor gene expression of the toluene dioxygenase and determine optimum conditions for gene expression both in controlled conditions and an environmental situation. The greatest potential of the use of such a strain is in pretreatment of waste as it has lots of advantages. It allows the on-line monitoring of gene expression and also the toxic effects of TCE and its metabolites on the system. This second attribute of the system can be seen in the fact that the intensity of the light response (Figure 22) is reproducible in the successive perturbations. This suggests that there was not a significant toxic effect of the TCE or its metabolites on the system as the light response level was not affected. Future studies will focus on the use of this strain to determine the minimum amount of gene expression to attain maximum TCE removal of the system and the use of gratuitous inducers to eliminate the competitive inhibition of the toluene.

3.5 Construction of *biphenyl-lux* Reporter Strains. Erickson and Mondello (1992) have reported two putative promoter regions for the *bph* operon. One of the regions is directly in front of the *bphA*1 gene and the other is in front of an Open-Reading-Frame (ORFO) that also

precedes *bphA*1. Two *bph-lux* plasmids were constructed in order to examined the response of this region to biphenyl (Figure 3). One *bph-lux* construct contains both the ORFO and *bph*A1 before the *lux* genes (*ORFObphA-lux*) and the other construct contains just the *bphA*1 gene in front of the *lux* genes (*bphA-lux*). In the *E. coli* host neither of these constructs produced light with or without the presence of biphenyl. When the *bph-lux* plasmids were transferred to *P. putida* PB2440 light was produced.

Light production experiments with the *P. putida* (*ORFObphA-lux*) and *P. putida* (*bphA-lux*) strains were performed using conditions used to effectively degrade PCBs by naturally occurring PCB degrading bacteria. In the strain *P. putida* (*ORFObphA-lux*) light increased as the cells grew in pyruvate and the addition of biphenyl resulted in higher light production (Figure 23). An increased light response was not seen with the addition of biphenyl in the *P. putida* (*bphA-lux*) strain. A similar increase in light production was seen with the addition of biphenyl for strain *P. putida*::TnPCB (*ORFObphA-lux*; Figure 24).

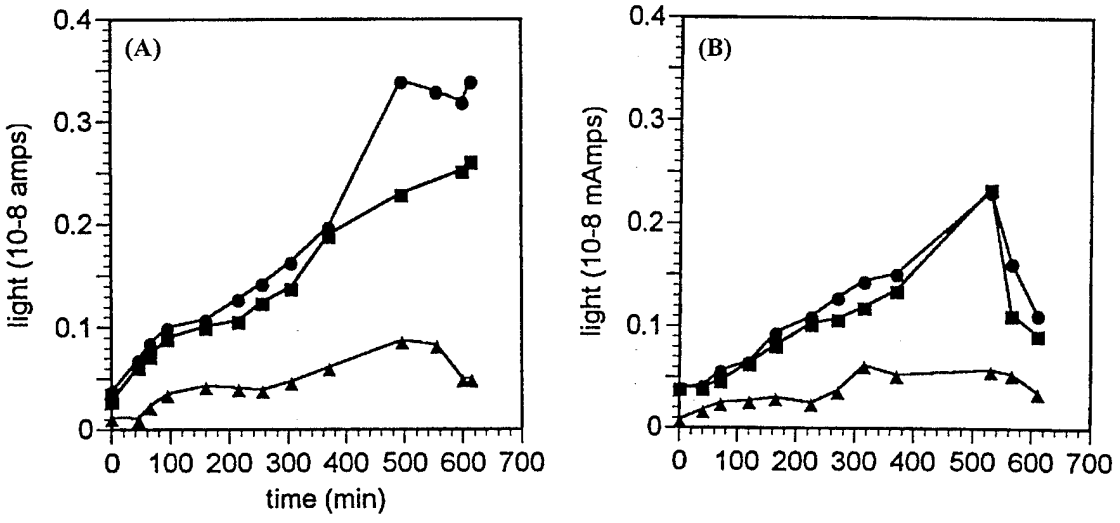

Figure 23. Light production by the strains *P. putida* (*ORFObphA-lux*) and *P. putida* (*bphA-lux*). Concentrations of the carbons sources were as follows: pyruvate (80 mM; ■), pyruvate + benzoate (80 mM; ▲), pyruvate + biphenyl (1%; •).

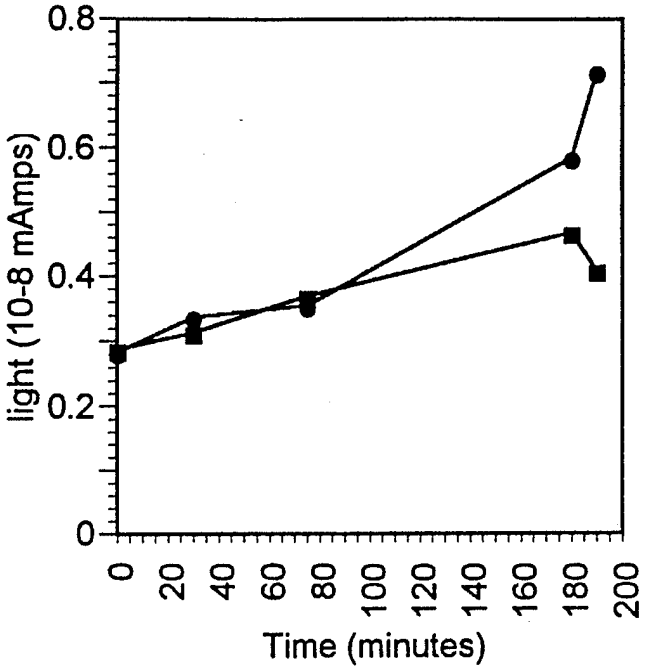

Figure 24. Light production by the strain *P. putida*::TnPCB (*OFObphA-lux*). Pyruvate (■); pyruvate + biphenyl (•).

Other bioluminescent reporter systems such as the *nah-lux* and the *tod-lux* systems appear to have stronger light responses to naphthalene and toluene respectively. Several factors may account for these results. First biphenyl has a low solubility so that the amount of biphenyl available to the cells may be low. Second, the nucleotide sequence of the P1P2 promoter region resembles the Sigma 54 type promoters. Genes with these types of promoters are often affected by carbon and nitrogen levels in the cells. In addition, pyruvate is an intermediate in the degradation of the biphenyl ring.

Benzoate was tested as a potential inducer of the *bph* promoter because it is a final end product of the upper biphenyl degradative pathway. When benzoate was used as the carbon source, light production was repressed compared to pyruvate in the strain *P. putida* (*ORFObphA-lux*; Figure 23). This result was unexpected because salicylate, the end product of the upper

pathway of the naphthalene serves as the inducer of the nah promoter. One explanation may be that the naphthalene and the salicylate pathways are linked in *Pseudomonas*, where as the PCB/biphenyl and the benzoate pathways are not linked and often occur in different bacterial species in a consortia (Pettigrew, 1990; Hickey, 1993).

3.6 Degradation of PAHs Mediated by a NAH Plasmid. The bacteria *P. fluorescens* strain 5R and its mutant 5RL were used in this investigation. Preliminary results obtained from TLC indicated that there are metabolites formed in the degradation of anthracene and phenanthrene by strain 5RL. Identification of metabolites are further analyzed by HPLC and GC/MS. Two degradation products, 2-hydroxy-3-naphthoic acid and 1-hydroxy-2-naphthoic acid, are recovered and identified as biochemical metabolites from the biotransformation of anthracene and phenanthrene, respectively. This is the first study providing direct biochemical evidences of the naphthalene plasmid degradative enzyme system being involved in the degradation of higher molecular weight PAHs other than naphthalene.

Characterization of metabolites formed from fluorene was also carried out by biotransformation experiments. A yellow color product was observed in the biotransformation study, however, the physical property the compound was not able to determine at present time. Preliminary results obtained from TLC plate suggest that there were accumulation of metabolites in the studies. Identification of metabolites were further analyzed by GC/MS. In neutral extraction, three metabolites were identified by GC/MS by comparison with the authentic standard or mass spectrum obtained from previous published paper. The compound 9-fluorenol (m/z=181; base peak) and 9H-fluoren-9-one (m/z=180; base peak) were identified with GC retention time of 13.4 and 13.5 minutes, respectively. The third metabolite had a GC retention

time of 7.8 minute and was identified as 1-indanone (2,3-dihydro-1H-inden-1-one) with a parent peak (M^+) of m/z=132 and a base peak of m/z=104. The metabolite, 1-indanone, was also detected in the transformation experiments by strain 5R. To our knowledge, this compound, 1-indanone, was never reported as an intermediate in the degradation of fluorene by either bacteria or fungi.

Another three metabolites with M.W. of 148 was detected by GC/MS in the neutral extraction with retention time of 12.5, 13.8, and 14.5 min, respectively. This compound could be converted from 1-indanone by strain 5R and an *E. coli* strain that containing *nahABCD* genes, and from the parent substrate, fluorene, by strain 5R, but not by *E. coli* strain. The identity of these metabolites is not determined at present time. The retention time of these compounds was different from 2-hydroxy-indanone 13.2 min), and 3-hydroxy-indanone (15.4 min; both have M.W. of 148) that formed from 1-indanone by naphthalene dioxygenase, and toluene dioxygenase (Resnick, 1994). This study suggested that the NAH plasmid present in strain 5R/5RL has the ability to mediate the degradation of fluorene and may possess a different metabolism pathway for fluorene. A further investigation on the identification of these metabolites is in progress.

A bioluminescent assay has been developed to ascertain whether the metabolite(s) produced from biotransformation of fluorene by the *nah* operon capable of interacting with the *nahR* gene product to induce the *nah* and *sal* operons. Bioluminescent values were obtained after one hour exposure. A T test of the data showed no significant difference between the negative control and the fluorene cultures. While the positive control (salicylate 10 mg/l) showed a significant bioluminescence response indicating induction of the *nah* and *sal* operons. These

results suggested that fluorene biotransformation does not provide metabolite(s) which can interact with the regulatory protein of the NAH system. It also suggests that the biotransformation of fluorene by NAH plasmid is a cometabolism process.

Dibenzofuran can be transformed rapidly by strain 5RL using the procedures described in the Materials and Methods. After dibenzofuran is added into 5RL growing culture, a yellow color formed within 30 min. The spectrophotometric characteristics of this yellow color product showed maximum absorptions at 427 and 359 nm, under neutral and basic conditions, and it shifted to maximum absorption at 397 nm, under acidic condition. It is interesting to note that these absorption characteristics change to 420, 358, and 307 nm, under neutral and basic conditions, and to 316 nm under acidic condition, after 22 hr. The residues obtained from transformation experiments were further analyzed by GC/MS. In neutral extracts, 2-dibenzofuranol (m/z at 184; base peak), 4H-1-benzopyran-4-one (chrome; m/z at 146), 3(2H)-benzofuranone (m/z at 134; base peak), and 2-hydroxy-benzenemethanol (salicyl alcohol; m/z at 124; with base peak at 78) were detected. In acid extracts, 2-hydroxy-benzoic acid (salicylate; m/z at 138 with base peak at 120) and its methyl ester (m/z at 152) were detected. All metabolites were identified by compared with mass spectrum from NIST Mass Spectral Library. A further characterization of these compounds are undertaken.

The results obtained from this study suggested a different degradation pathway of dibenzofuran may present in strain 5RL. The benzene ring was the initial target that cleaved by 5RL and led to form 2-dibenzofuranol and 3(2H)-benzofuranone which different from other reported bacteria that break the central ring first and yield 2,2',3-trihydroxybiphenyl (Fortnagel, 1990; Strubel, 1991; Happe, 1993). This observation was consisted with the degradation of

fluorene by 5RL that the initial attack was on the six-ring. However, this hypothesis needs further confirmed by isolation and identification of these metabolites.

Strain 5RL also showed ability to degrade dibenzo-*p*-dioxin, however, the identities of the transformation products are not determined at present time. In addition, strain A5 also showed ability to transformed dibenzo-*p*-dioxin, and a potential metabolite was determined as salicyl alcohol by comparison with NIST Mass Spectral Library.

The above results suggested that the strain 5R/5RL has a great diversity of its degradative capability to degrade different PAHs. In other words, the NAH plasmid plays important role in the degradation of 3-ring PAHs and cyclic biaryl ethers, and its metabolic pathway may serve as a core pathway for the microbial degradation of hazard waste in the environment.

3.7 Naphthalene Degradation Through the Gentisate Pathways by *Burkholderia cepacia* Strain JS150. *Pseudomonas* sp. strain JS1 has mucoid phenotype, and has a broad substrate specificity on the oxidation of aromatic hydrocarbons (Spain, 1990). A non-mucoid mutant, JS150, was isolated and retained all known degradative capabilities of JS1, including naphthalene and salicylate (Haigler, 1992). DNA-DNA hybridization under high stringency conditions revealed significant homology between JS150 DNA and the *nahA* (naphthalene dioxygenase) gene of naphthalene catabolic plasmid NAH7. However, JS150 did not hybridize to *nahG* (salicylate hydroxylase gene) or *nahR* (positive regulatory gene) probes. This results indicated that the naphthalene pathway of JS150 may be regulated by a different system than the naphthalene pathway of NAH7. It is also possible that JS150 has a *nahR* homologue which is further diverged from *nahR* than the *nahR* homologues typically found on naphthalene catabolic plasmids, although it seems unlikely that the sequences of *nahA* and *nahR* in JS150 would

diverge from their NAH7 counterparts to a significantly different degree. Haigler *et al* (1992) showed that naphthalene oxidation by JS150 is inducible by growth on naphthalene or salicylate. Therefore, JS150 must have one or more functional naphthalene regulatory gene(s).

In the biotransformation experiments, both gentisate and salicylate were detected by GC/MS in the supernatant of naphthalene-grown culture. Furthermore, gentisate was discovered by HPLC and GC/MS in the salicylate-grown culture and gentisate dioxygenase activity was detected in salicylate-grown cell-free extract. Further enzyme assays showed that catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities were not detected in the cell-free extract of salicylate-grown culture. Complete degradation of gentisate by cell-free extract of salicylate-grown JS150 was shown to be dependent on the presence of reduced glutathione. The above results strongly suggested that JS150 oxidizes naphthalene to salicylate, then further degradation *via* a reduced glutathione-dependent gentisate pathway. The ability of JS150 to grow on potential gentisate pathway substrates was examined (Table 7). It is interesting to note that among the large number of aromatic compounds can serve as growth substrates for JS150 (Haigler, 1992), only naphthalene and salicylate were degraded through the gentisate pathway. There have been no previous reports of a strain using the gentisate pathway but being unable to use exogenous gentisate as a growth substrate. Another compound, *m*-hydroxybenzoate which is degraded through the gentisate pathway by some bacteria, was found to be degraded through protocatechuate *ortho* cleavage in JS150. Anthranilic acid, another potential gentisate pathway substrate, was also shown not to be degraded through the gentisate pathway in JS150. The actual catabolic pathway for anthranilic acid was not identified.

Table 7. Substrate specificity study of JS150 *via* the gentisate pathway.

Substrate	Growth	Ring-cleavage pathway	Reference
Naphthalene	+	MPI [a]	This work, (Haigler, 1992)
Salicylate	+	MPI	This work, (Haigler, 1992)
Gentisate	–	MPI	This work
m-hydroxybenzoate	+	PCA 3,4[b]	This work
anthranilate	+	?[c]	This work, (Haigler, 1992)
4-chlorosalicylate	–	ND[d]	This work
3-methylsalicylate	–	ND	This work
4-methylsalicylate	–	ND	This work
Tryptophan	+	?	This work

[a] Gentisate pathway, maleylpyruvate isomerase branch.
[b] Protocatechuate *ortho* cleavage.
[c] Ring cleavage pathway present, but unknown.
[d] Occurrence of ring cleavage not determined

The results of the research reported here brought to seven the number of cleavage pathways possessed by strain JS150. These are: 1) the naphthalene upper pathway, 2) the gentisate pathway, 3) the catechol *ortho* cleavage pathway (Haigler, 1992), 4) at least one catechol *meta* cleavage pathway (Haigler, 1992), 5) the modified *ortho* cleavage pathway (Spain, 1987), 6) the protocatechuate *ortho* cleavage pathway, and 7) the unidentified pathway through which anthranilate is degraded. This is greater than the number required for the metabolism of the substrates JS150 is known to degrade. The presence of so many ring cleavage pathways in JS150 makes JS150 a good strain in which to investigate the relative efficiencies of few versus many ring cleavage pathways for the simultaneous degradation of multiple aromatic substrates.

3.8 Degradation of Chlorinated Hydrocarbons by *Alcaligenes eutrophus* A5. A PCB degrader, *A. eutrophus* A5, was used in this study. Biotransformation of DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) strain A5 was demonstrated by analysis of ethyl acetate extracted

products from resting cell cultures. The GC/MS analysis of the neutral extracts from the cultures revealed a metabolite with retention times at 19.80 and 19.55 min. which showed identical mass spectrum. These metabolites had a parent peak (M^+) of m/z 370 and a base peak of m/z 251 and was tentatively determined to be a hydroxy-DDT because it had a molecular weight of 370, which is 16 more than DDT (M.W. 354). The ionization fragment of m/z 251 indicated the loss of -CCl_3. This ionization pattern consisted of three sequential ionization peaks, after the parent peak (m/z 370), at m/z 335, 298 and 262 which corresponds to the loss of three chloride ions. The remaining ion fragment m/z 251 could be $C_{13}H_9OCl_2$ (M.W. 251). These metabolites are considered to be hydroxyl substituents at *meta-* and *ortho-* positions on the phenyl ring resulting from dehydration of the dihydrodiol metabolite (Focht, 1970; Furukawa, 1978). Metabolites were not detected in extractions performed on the biotic and abiotic controls. This result suggested that the hydroxylation occurred at the *ortho-* and *meta*-positions on the aromatic ring. In addition, a yellow ring-cleavage product was observed in the supernatant, and showed a maximum absorbance at 402 nm with, under acidic and basic conditions, similar spectrophotometric characteristics as aromatic ring *meta*-cleavage products.

In the acid extract, one intermediate with a GC retention time of 8.0 min. and a molecular ion peak of m/z 156 and a base peak of m/z 139 (M^+-OH) was detected. This product was identified as 4-chlorobenzoic acid (4-CBA) by comparison to authentic standard which suggested that 4-CBA is a ring-cleavage product formed from DDT by strain A5. The 4-CBA was also detected by TLC radiochemical scanning in sample from mineralization experiments by comparison of R_f values of ^{14}C-DDT intermediates and an authentic standard.

This study indicates that DDT appears to be oxidized by a dioxygenase in *Alcaligenes eutrophus strain* A5 and the products of this oxidation are subsequently subjected to ring fission to eventually yield 4-chlorobenzoic acid, via a *meta*-cleavage pathway, as a major stable intermediate.

In addition to *o,p'*-DDT, strain A5 also showed degradative ability to transform *o,p'*-DDT, *p,p'*- and *o,p'*-DDE, and *p,p'*- and *o,p'*-DDD. The yellow color was observed in all six compounds, under the neutral and basic conditions. It is interesting to note that the yellow color was not able to recover after acidification. The characteristic absorption maximum of the yellow ring-fission products formed from different substrate are showing in Table 8. In a 7-day transformation of *p,p'*-DDT, a potential metabolite was determined as salicyl alcohol (m/z at 124 with base peak at 78) in the neutral extract by compared with NIST Mass Spectral Library. A compound with a molecular ion peak of m/z at 156 was detected in both acid extracts of *p,p'*- and *o,p'*-DDE transformation study. These results suggested chlorobenzoates may serve as possible metabolites in both cases. However, the true identity of the chlorobenzoates and other

Table 8. Characteristic absorption maximum of the yellow ring-fission products formed from different chlorinated compounds by strain A5.

Substrate	**Absorption Maxima (nm)**		
	Acid	**Neutral**	**Base**
p,p'-DDT	-	402	402
o,p'-DDT	-	407	407
p,p'-DDE	-	402	402
o,p'-DDE	-	406	406
p,p'-DDD	-	410	412
o,p'-DDD	-	409	412

-: no detectable peak.

metabolites is not determined at present time.

In the transformation experiment of chlorobenzilate by strain A5, two potential metabolites were determined as 4,4'-dichlorobenzophenone (m/z at 250) and salicyl alcohol (m/z at 124) by comparison with the mass spectra from NIST library. The latter compound, salicyl alcohol, was also detected when hexachlorophene used as a substrate. Strain A5 also showed ability to degrade dicofol to 4,4'-dichlorobenzophenone and 4-chlorobenzoate as determined by comparison the mass spectra. In addition, strain A5 exhibited different degree of degradative capability on the degradation of bifenox, methoxychlor, and perthane that determined by the GC profiles of the transformation experiments. However, the identity of those compounds are not resolved at present time.

The GC/MS results indicated that salicyl alcohol, 4,4'-dichlorobenzophenone and, chlorobenzoate are common metabolites formed from DDT structure-like substrates. This investigation also suggested that strain A5 exhibits a great diversity degradative capability on the degradation of DDT structure-like compounds and PCBs. *In situ* bioremediation on chlorinated compounds in the future by strain A5 is predictable and feasible because its metabolic versatility and the knowledge we have at the biochemical (current study) and molecular level (Mergeay, 1993).

3.9 Analysis of PCB Degradation. In addition to degrading biphenyl and chlorobiphenyl, *Alcaligenes eutrophus* A5 was able to cometabolize 16 of the congener peaks from Aroclor 1242 (Table 9). The range of PCB congener metabolism is better than many biphenyl degrading bacteria but is not as wide as the strain *Pseudomonas* sp. LB400 (Mondello, 1989).

Table 9. Degradation of Aroclor 1242 in resting cell assays by *Alcaligenes eutrophus* A5.

Peak #	Congener Identification	% Degradation[a]
1	2	nd
2	2,2'; 2,6	30
3	2,4; 2,5	100
4	2,3'	100
5	2,3; 2,4'	100
6	2,6,2'	0
7	2,5,2'	0
8	2,4,2'; 4,4'	0
9	2,3,6; 2,6,3'	45
10	2,3,2'; 2,6,4'	25
11	2,5,3'	60
12	2,4,3'	100
13	2,5,4'	60
14	2,4,4'	0
15	2,3,4; 2,5,2',6'	80
16	2,3,4'; 2,4,2',6'	90
17	2,3,6,2'	0
18	2,3,2',6'	nd
19	2,5,2',5'	0
20	2,4,2',5'	0
21	2,4,2',4'	0
22	2,4,5,2'	0
23	2,3,2',5'	0
24	3,4,4'; 2,3,2',4'	0
25	2,3,4,2'; 2,3,6,4'; 2,6,3',4'	0
26	2,3,2',3'	15
27	2,4,5,4'	20
28	2,5,3',4'	0
29	2,4,3',4'; 2,3,6,2',5'	15
30	2,3,6,2',4'	20
31	2,3,3',4'; 2,3,4,4'	80
32	2,3,6,2',3'; 2,3,5,2',5'	0

33	2,3,5,2',4'; 2,4,5,2',5'	0
34	2,4,5,2',4'	nd
35	2,4,5,2',3'; 2,3,5,6,2',6'	0
36	2,3,4,2',5'	0
37	2,3,4,2',4'	0
38	2,3,6,3',4'; 3,4,3',4	0
39	2,3,4,2',3	0
40	2,3,6,2',4',5'; 2,4,5,3',4'	0
41	2,3,4,3',4'; 2,3,4,2',3',6'	0

[a]Percent degradation determined by comaparison to killed controls.
nd- not detected in this Aroclor 1242 standard.

To compare the relationship of the PCB degrading genes in A5 to other known PCB degrading bacteria, colony hybridization experiments of A5 with gene probes from biphenyl operon were performed (Layton, 1994). The *bphBC* gene probe from *Pseudomonas pseudoalcaligenes* KF707 and the *bphABC* gene probe from *Alcaligenes eutrophus* ENV307 did not hybridize to A5. A *bphBC* gene probe from *Pseudomonas testosteroni* B-356 hybridized weakly to A5. This indicates that the DNA sequences of the *bph* genes from A5 differ from other characterized biphenyl/PCB degrading bacteria such as *Pseudomonas* sp. LB400.

3.10 Dehalogenation of 4-Chlorobenzoate. When the strain *Alcaligenes* sp. A5 was originally isolated it was capable of mineralizing 4-CB to CO_2. Subsequently, A5 lost its ability to mineralize 4-CB to CO_2 but retained its ability to produce the metabolite 4-CBA. The loss in ability to mineralize 4-CB was associated with a change in plasmid size from 50 Mdal to 35 Mdal (Shields, 1985; Burlage, 1990). Two other bacteria isolated from the same location, 1C1 (Pettigrew, 1990) and ALP83 (Layton,1992) have the capability of mineralizing 4-CB to CO_2 and contain plasmids similar to pSS50 from A5 with the exception of an additional 10 - 13 kb

inserts. The plasmids from 1C1 and ALP83 were designated as pSS60 and pSS70 respectively and belong the to incompatibility group P1, ß subgroup (Burlage, 1990).

Restriction enzyme mapping indicated that plasmids pSS50, pSS60 and pSS70 all contain 2.2 kb inverted repeat regions (Layton, 1992). In pSS60 and pSS70 these inverted repeats flank the unique DNA fragment. DNA hybridization was used to demonstrate that the unique DNA fragments in pSS60 and pSS70 cross hybridized.

In strain ALP83, pSS70 was unstable and the unique DNA fragment carried on pSS70 was easily lost. When colonies from ALP83 were hybridized with pSS50 and a cloned fragment from pSS60 (B-3) two types of colonies were detected: 1) those that probed positive with both pSS50 and B-3 and 2) those that probed positive only with pSS50. Individual colonies of strain ALP83 obtained by selection of spontaneous mutants cultures on YEPG-rifampicin (50µg/ml) agar plates. Plasmid DNA from these rifampicin mutants migrated as one or two bands after electrophoresis through an agarose gel. The higher band was designated as pSS70 and the lower band which comigrated with pSS50 was designated as pSS65.

Chloride release from 4-CBA was examined in strains carrying pSS50, pSS65 and pSS70 in order to correlate dechlorination of 4-CBA with the unique fragment carried on pSS70. *Arthrobacter* sp. strain TM-1 was used as a positive control that dechlorinated 4-CBA without any accumulation of intermediates (Layton, 1992). *Alcaligenes* sp. strain A5 (pSS50) and AL3019 (pSS65) did not dechlorinate 4-CBA whereas ALP83 (pSS70) and AL3007 (pSS70) did dechlorinate 4-CBA with an accumulation of 4-hydroxybenzoic acid (4-HBA).

These results demonstrated the 4-CB degrading stain ALP3 can degrade 4-CBA to 4-HBA and that the dehalogenase activity is correlated with a 10 kb fragment carried on the

plasmid pSS70. However, ALP83 did not metabolize 4-HBA, so 4-CBA alone does not provide a good growth substrate.

4. CONCLUSION

The correlation between bioavailability and biodegradative capability in the environment has always been a puzzle for bioremediation. Furthermore, the detection of biodegradative activities *in situ* also has hampered biological site characterization. All of these due to lack of proper tool(s) or method(s) that can be applied readily, specifically, and feasibly to the environmental pollutants. However, the development and application of bioluminescent reporter strains for continuously real-time monitoring the relationship between bacterial degradative activities and bioavailability of environmental pollutants were examined in this study. The results obtained from this investigation suggested that bioluminescent reporters can provide continuous, and precise insight information on both molecular and physiological level. The more important is that these bioreporters will not interrupt and compete with indigenous bacteria.

The versatility of the catabolic capability on the degradation of different higher molecular PAHs by a NAH plasmid-mediated metabolism was also examined. The results obtained in this study indicated that the NAH plasmid plays an important role on the biodegradation of PAHs. Furthermore, the naphthalene degradation pathway serves an essential route for the study of bacterial degradation pathway on PAHs. A similar observation was also obtained on the bacterial degradation of chlorinated compounds by a PCB-degrader. These results suggested the existence of common degradation pathway(s) used by bacteria on the degradation of environmental hazardous wastes.

The major outcomes of this investigation are listed as below:

(1) Both microbial and genetic ecology characterization of bacterial distributions in PAH contaminated soils and creosote-contaminated soils.

(2) Demonstration of the naphthalene degradative genes were presented in all seven PAH-contaminated soils, which used in this study, *via* positiv hybridization with *nahA* gene probe.

(3) Four non-NAH genotype naphthalene-degraders were identified and they may serve as sources for new gene probes for monitoring distribution of indigenous bacteria population.

(4) Application of *nah-lux* bioreporter for real-time monitoring environmental pollutants' bioavailability and bioremediation process(s) *in situ*.

(5) Development of two new bioreporter strains, *tod-lux* and *biphenyl-lux*, for monitoring degradation activities of TCE and biphenyl *in situ*, respectively.

(6) Plasmid stability and reporter strain maintenance.

A. Plasmid stability:

- The segregational stability of pUTK21 was the best in 5RL with 60% of the culture retaining pUTK21 for 98 generations under non-selective conditions of continuous culture.
- The pUTK21 plasmid was segregationally unstable in HK44 under non-selective continuous culture conditions. The retention of pUTK21 in HK44 dropped to <1% of the total culture after 39 generations.

- The segregationally stability of pUTK9 was shown to range from 67-95% in RB1351 under non-selective continuous culture conditions.
- None of the reporter plasmid were maintained as well as the wild type naphthalene biodegradation plasmid pKA1 (100% maintenance).

B. Strain maintenance:

- Reporter strain 5RL is not maintained under competitive continuos culture conditions
- Reporter strain RB1351 was maintained at low levels under competitive continuous culture conditions.
- Reporter strain HK44 was maintained under competitive continuous culture conditions when either allowed to attain steady state before competitors were added or when added at cell concentrations higher than the competitor.
- None of the reporter strains were as competitive as the wild type strain 5R.

(7) Demonstration the degradation of naphthalene by *Burkholderia cepacia* JS150 was through the gentisate pathway.

(8) Demonstration the catabolic versatility of NAH plasmid that present in strain 5R/5RL on the degradation of different PAHs, such as anthracene, dibenzofuran, fluorene, and phenanthrene etc.

(9) Demonstaration the *bph* genes in strain A5 showed a great diversity of degradative capabilities on the degradation of chlorinated compounds, such as DDT, DDE, DDD, and other chlorinated pesticides.

5. REFERENCE

Ahmad, D., R. Masse, and M. Sylvestre. 1990. Cloning and expression of genes involved in 4-chlorobiphenyl transformation by *Pseudomonas testosteroni*: homology to polychlorobiphenyl-degrading genes in other bacteria. Gene **86:**53-61.

Ahmad, D., M. Sylvestre, and M. Sondossi. 1991. Subcloning of *bph* genes from *Pseudomonas testosteroni* B356 in *Pseudomonas putida* and *Escherichia coli*: evidence for dehalogenation during initial attack on chlorobiphenyls. J. Bacteriol. **57:**2880-2887.

Alam, K.Y., M.J. Worland, and D.P. Clark. 1990. Analysis and molecular cloning of genes involved in thiophene and furan oxidation by *E. coli*. Appl. Biochem. Biotechnol. **24-25:**843-856.

Ames, B.N. 1971. The detection of chemical mutagens with enteric bacteria. *In* Chemical Mutagens, Principles and Methods for their Detection, (Hollaender A, ed.). Plenum Press, New York. p.267-282.

Ames, B.N. 1972. A bacterial system for detecting mutagens and carcinogens. *In* Mutagenic Effects of Environmental Contaminants, (Sutton HE and Harris MI, eds.). Academic Press, New York. p.57-66.

Asturias, J.A., L.D. Eltis, M. Prucha, and K.N. Timmis. 1994. Analysis of three 2,3-dihydroxybiphenyl 1,2-dioxygenases found in *Rhodococcus globerulus* P6. Identification of of a new family of extradiol dioxygenases. J. Biol. Chem. **269:**7807-7815.

Bagdasarian, M.R.L., B. Ruckert, F.C.H. Franklin, M.M. Bagdasarian, J. Frey, and K.N. Timmis. 1981. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene **16:**237-247.

Baldwin, T.O., J. H. Devine, R. C. Heckel, J. -W. Lin, and G. S. Shadel. 1989. The complete nucleotide sequence of the *lux* region of *Vibrio Fischeri* and the *lux*ABN region of *Photobacterium Leiognathi* and the mechanism of control of bacterial bioluminescence. J. Chemiluminescence and Bioluminescence. **4:**326-341.

Beckman Instruments. 1978. Microtox model 2055 toxicity analyzer system. Carlsbad, CA, USA.

Bedard, D.L., R. Unterman, L.H. Bopp, M.J. Brennan, M.L. Harbe, and C. Johnson. 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51**:761-768

Bedard, D.L., R.E. Wagner, M.J. Brennan, M.L. Harbel, and J.F. Brown, Jr. 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53**:1094-1102.

Bitton, G., B. Koopman, and O. Agami. 1992. MetPad™: a bioassay kit for the rapid assessment of heavy metal toxicity in waste water. Water Environ. Res. **64:**834-836.

Burlage, R.S., L.A. Bemis, A.C. Layton, G.S. Sayler, and F. Larimer. 1990a. Comparative genetic organization in incompatibility group P degradative plasmids. J. Bacteriol. **172:**6818-6825.

Burlage, R.S., A. Heitzer, and G.S. Sayler. 1992. Bioluminescence: a versatile bioreporter for monitoring bacterial activity. BFE **9:**704-709.

Burlage, R.S., G.S. Sayler, and F. Larimer. 1990b. Monitoring of naphthalene catabolism by bioluminescence with nah-lux transcriptional fusions. J. Bacteriol. **172:**4749-4757.

Cerniglia, CE. 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation **3:**351-368.

Chakrabarty, A.M., G. Chou, and I.C. Gunsalus. 1973. Genetic regulation of octane dissimulation plasmid in *Pseudomonas*. Proc. Natl. Acad. Sci. USA **70:**1137-1140.

Chaudhry, G.R., and S. Chapalamadugu. 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. **55:**59-79.

Church, G.M., and W. Gilbert. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991-1995.

Coco, W.M., R.K. Rothmel, S. Henikoff, and A.M. Chakrabarty. 1993. Nucleotide sequence and initial functional characterization of the *clcR* gene encoding a *LysR* family activator of the *clcABD* chlorocatechol operon in *Pseudomonad putida*. J. Bacteriol. **175:**417-427.

Commandeur, L.C.M., and J.R. Parsons. 1990. Degradation of halogenated aromatic compounds. Biodegradation **1:**207-220.

Corbisier, P., L. Diels, G. Nuyts, W. Baeyens, and M. Mergeay. 1992. Copper resistance in large plasmids of *Alcaligenes eutrophus*. Abstracts of ASM General Meeting, May 1990, New Orleans, USA.

Corbisier, P., L. Diels, D. van der Lelie, and M. Mergeay. 1993a. Proceedings of the Sixth International Symposium, toxicity assessment and on-line monitoring. May 10-14, 1993, Berlin, Germany. p. 58.

Corbisier, P., G. Ji, G. Nuyts, M. Mergeay, and S. Silver. 1993b. *LuxAB* fusions with the arsenic and cadmium resistance operons of *Staphylococcus aureus* plasmid pI258. FEMS Microbiol. Lett. **110:**231-238.

Dabrock, B., M. Kebeler, B. Averhoff, and G. Gottschalk. 1994. Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 that encodes isopropylbenzene and trichloroethylene catabolism. Appl. Environ. Microbiol. **60:**853-860.

Dagley, S. 1986. The biology of *Pseudomonas*. New York. Academic Press, Inc., p.527-555.

De Vevey, E., G. Bitton, D. Rossel, L.D. Ramos, G.L. Munguia, and J. Tarradellas. 1993. Concentration and bioavailability of heavy metals in sediments in lake Yojoa (Honduras). Bull. Environ. Contam. Toxicol. **50:**253-259.

de Zwart, D., and W. Sloof. 1983. The Microtox as an alternative assay in the acute toxicity assessment of water pollutants. Aquat. Toxicol. **4:**129-138.

Denome, S.A., D.C. Stanley, E.S. Olson, and K.D. Young. 1993. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. **175:**6890-6901.

Don, R.H., and J.M. Pemberton. 1985a. Genetic and physical map of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pJP4. J. Bacteriol. **161:**466-468.

Don, R.H., A.J. Wieghtman, H.H. Knackmuss, and T.N. Timmis. 1985b. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. **161:**85-90.

Doten, R.C., K.-L. Ngai, D.J. Mitchell, and L.N. Ornston. 1987. Cloning and genetic organization of the *pca* gene cluster from *Acinetobacter calcoaceticus*. J. Bacteriol. **169:**3168-3174.

Dunn, N.W., and I.C. Gunsalus. 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bacteriol. **114:**974-979.

Eaton, R.W., and K.N. Timmis. 1986. Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in *Pseudomonas putida* RE204. J. Bacteriol. **168:**123-131.

Ensley, B.D., B.J. Ratzkin, T.D. Osslund, M.J. Simon, L.P. Wackett, and D.T. Gibson. 1983. Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. Science **222:**167-169.

Erickson, B.D., and F.J. Mondello. 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in Pseudomonas strain LB400. J. Bacteriol. **174:**2903-2912.

Fennewald, M., S. Benson, M. Oppici, and J. Shapiro. 1979. Insertion element analysis and mapping of the *Pseudomonas* plasmid *alk* regulation. J. Bacteriol. **139:**940-952.

Festl, H.W.L., and K.H. Schleifer. 1986. DNA hybridization probe for the *Pseudomonas fluorescens* group. Appl. Environ. Microbiol. **52:**1190-1194.

Focht, D.D., and M. Alexander. 1970. DDT metabolites and analogs. Ring fission by *Hydrogenomonas*. Science **170:**91-92.

Foght, J.M., and D.W.S. Westlake. 1988. Degradation of polycyclic aromatic hydrocarbons and aromatic heterocyclics by *Pseudomonas* species. Can. J. Microbiol. **34:**1135-1141.

Foght, J.M., and D.W.S. Westlake. 1991. Cross hybridization of plasmid and genomic DNA from aromatic and polycyclic aromatic hydrocarbon degrading bacteria. Can. J. Microbiol. **37:**924-932.

Fortnagel, P., H. Harms, R.-M. Wittich, S. Krohn, N. Meyer, V. Sinnwell, H. Wilkes, and W. Francke. 1990. Metabolism of dibenzofuran by *Pseudomonas* sp. strain HH69 and the mixed culture HH27. Appl. Environ. Microbiol. **56:**1148-1156.

Frantz, B., and A.M. Chakrabarty. 1986. The biology of *Pseudomonas*. New York. Academic Press, Inc. p.295-323.

Frantz, B., and A.M. Chakrabarty. 1987. Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. Proc. Natl. Acad. Sci. USA **84:**4460-4464.

Fredrickson, J. K., F.J. Brockman, D.J. Workman, S.W. Li, and T.O. Stevens. 1991. Isolation and characterization of a subsurface bacterium capable of growth on toluene, naphthalene, and other aromatic compounds. Appl. Envron. Microbiol. **57:**796-803.

Friello, D.A., J.R. Mylroie, D.T. Gibson, J.E. Rogers, and A.M. Chakrabarty. 1976. XYL, a nonconjugative xylene degradative plasmid in *Pseudomonas* Pxy. J. Bacteriol. **127:**1217-1224.

Furukawa, K. 1994a. Molecular genetics and evolutionary relationship of PCB-degrading bacteria. Biodegradation **5:**289-300.

Furukawa, K., K. Tonomura, and K. Kamibayashi. 1978. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. **35:**421-428.

Gibson, D.T. 1977. Fate and effects of petroleum hydrocarbons in marine organisms and ecosystems. New York, Pergamon Press. p.36-46.

Gibson, D.T. (ed.) 1984a. Microbial Degradation of Organic Compounds. New York. Marcel Dekker, Inc.

Gibson, D.T., and V. Subramanian. 1984b. Microbial degradation of organic compounds. New York, Marcel Dekker, Inc., p.181-252.

Green, J., and H. Dalton. 1989. Substrate specificity of soluble methane monooxygenase. J. Biol. Chem. **264:**17698-17703.

Gunsalus, I.C. 1985. Notes on metabolic plasmid organization and expression. *In* Plasmid in bacteria. New York. Plenum Press, p.687-706.

Haigler, B.E., C.A. Pettigrew, and J.C. Spain. 1992. Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. **58**:2237-2244.

Happe, B., L.D. Eltis, H. Poth, R. Hedderich, and K.N. Timmis. 1993. Characterization of 2,2',3-trihydroxybiphenyl dioxygenase, an extradiol dioxygenase from the dibenzofuran- and dibenzo-*p*-dioxin-degrading bacterium *Sphingomonas* sp. strain RW1. J. Bacteriol. **175:**7313-7320.

Harder, P.A., ans D.A. Kunz. 1986. Characterization of the OCT plasmid encoding alkane oxidation and mercury resistance in *Pseudomonas putida*. J. Bacteriol. **165:**650-653.

Harker, A.R., and Y. Kim. 1990. Trichloroethylene degradation by two independent aromatic-degrading pathways in *Alcaligenes eutrophus* JMP134. Appl. Environ. Microbiol. **56:**1179-1181.

Hayase, N., K. Taira, and K. Furukawa. 1990. *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. J. Bacteriol. **172:**1160-1164.

Heitzer, A., K. Malachowsky, J.E. Thonnard, P.R. Bienkowski, D.C. White, and G.S. Sayler. 1994. Optical biosensor for environmental on-line monitoring of naphthalene and salicylate bioavailability with an immobilized bioluminescent catabolic reporter bacterium. Appl. Environ. Microbiol. **60:**1487-1494.

Heitzer, A., O.F. Webb, J.E. Thonnard, and G.S. Sayler. 1992. Specific and quantitative assessment of naphthalene and salicylate bioavailability by using a bioluminescent catabolic reporter bacterium. Appl. Environ. Microbiol. **58:**1839-1846.

Hickey, W.J., D.B. Searles, and D.D. Focht. 1993. Enhanced mineralization of polychlorinated biphenyls in soils inoculated with chlorobenzoate-degrading bacteria. Appl. Environ. Microbiol. **59:**1194-1200.

Holmes, B., R.J. Owen, A. Evans, H. Malnick, and W.R. Willcox. 1977. *Pseudomonas paucimobilis*, a new species isolated from human clinical specimens, the hospital environment, and other sources. Intl. J. Sys. Bacteriol. **27:**133-146.

Holmes, D.S., and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193-197.

Hyman, M.R., I.B. Murton, and D.J. Arp. 1988. Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes. Appl. Environ. Microbiol. **54:**3187-3190.

Hyman, M.R., A.W. Sansome-Smith, J.H. Shears, and P.M. Wood. 1985. A kinetic study of benzene oxidation to phenol by whole cells of *Nitrosomonas europaea* and evidence for the further oxidation of phenol to hydroquinone. Arch. Microbiol. **43:**302-306.

Janssen, D.B., J.R. van der Ploeg, and F. Pries. 1994. Genetics and biochemistry of 1,2-dichloroethane degradation. Biodegradation **5:**249-257.

Jones, R.D., and R.Y. Morita. 1983. Carbon monoxide oxidation by chemolithotrophic ammonium oxidizers. Can. J. Microbiol. **29:**1545-1551.

Ka, J.O., and J.M. Tiedje. 1994b. Integration and excision of a 2,4-dichlorophenoxyacetic acid-degradative plasmid in *Alcaligenes paradoxus* and evidence of its natural intergeneric transfer. J. Bacteriol. **176:**5284-5289.

Keener, W.K., and D.J. Arp. 1994. Transformations of aromatic compounds by *Nitrosomonas europaea.* Appl. Environ. Microbiol. **60:**1914-1920.

Khan, A.A., and S.K. Walia. 1991. Expression, localization, and functional analysis of polychlorinated biphenyl degradation genes *cbpABCD* of *Pseudomonas putida.* Appl. Environ. Microbiol. **57:**1325-1332.

Kikuchi, Y., Y. Nagata, M. Hinata, M. Kimbara, M. Fukuda, K. Yano, and M. Takagi. 1994a. Identification of the *bphA4* gene encoding ferredoxin reductase involved in biphenyl and polychlorinated biphenyl degradation in *Pseudomonas* sp. strain KKS102. J. Bacteriol. **176:**1689-1694.

Kikuchi, Y., Y. Yasukochi, Y. Nagata, M. Fukuda, and M. Takagi. 1994b. Nucleotide sequence and functional analysis of the *meta*-cleavage pathway involved in biphenyl and polychlorinated biphenyl degradation in *Pseudomonas* sp. strain KKS102. J. Bacteriol. **176:**4269-4276.

Kim, Y., T. Ayoubi, and A. Harker. 1994. 94th General Meeting Amer. Soc. Microbiol., May 23-27. Las Vegas, NV., p.307.

Kimbara, K.T., T. Hashimoto, M. Fukuda, T. Koana, M. Takagi, M. Oishi, and K. Yano. 1989. Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. J. Bacteriol. **171:**2740-2747.

King, J.M.H., P.M. DiGrazia, B. Applegate, R. Burlage, J. Sanseverino, P. Dunbar, F. Larimer, and G.S. Sayler. 1990. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. Science **249:**778-781.

King, J.M.H., J. Sanseverino, P.M. Digrazia, B.M. Applegate, G.S. Sayler, and J.W. Blackburn. 1991. Molecular bioanalytical methods for monitoring PAH biodegradation in Manufactured gas plant soils, Final report, Vol. II, Gas Research Institute, Chicago.

Kiyohara, H., K. Nagao., and K. Yano. 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl. Environ. Microbiol. **43:**454-457.

Kiyohara, H., S. Torigoe, N. Kaida, T. Asaki, T. Iida, H. Hayashi, and N. Takizawa. 1994. Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. J. Bacteriol. **176:**2439-2443.

Kuhm, A.E., A. Stolz, and H.-J. Knackmuss. 1991. Metabolism of naphthalene by the biphenyl-degrading bacterium *Pseudomonas paucimobilis* Q1. Biodegradation **2:**115-120.

Kukor, J.J., and R.H. Olsen. 1990. Molecular cloning, characterization, and regulation of a *Pseudomonas pickettii* PKO1 gene encoding phenol hydroxylase and expression of the gene in *Pseudomonas aeruginosa* PAO1c. J. Bacteriol. **8:**4624-4630.

Kunz, D.A., and P.J. Chapman. 1981. Isolation and characterization of spontaneously occurring TOL plasmid mutants of *Pseudomonas putida* HS1. J. Bacteriol. **146:**952-964.

Lackey, L.W., T.J. Phelps, P.R. Bienkowski, and D. C. White. 1993. Biodegradation of chlorinated aliphatic hydrocarbon mixtures in a single-pass packed-bed reactor. Appl. Biochem. Biotech. **39/40:**701-713.

Lajoie, C.A., A.C. Layton, and G.S. Sayler. 1994. Cometabolic oxidation of polychlorinated biphenyl in soil with a surfactant-base field application vector. Appl. Environ. Microbiol. **60:**2826-2833.

Layton, A.C., C.A. Lajoie, J.P. Easter, R. Jernigan, M.J. Beck, and G.S. Sayler. 1994. Molecular diagnostics for polychlorinated biphenyl degradation in contaminated soils, p.407-422. *In* the Annals of the New York Academy of Sciences, Vol. 72: Recombinant DNA technology II.

Layton, A.C., J. Sanseverino, W. Wallace, C. Corcoran, and G.S. Sayler. 1992. Evidence for 4-chlorobenzoic acid dehalogenation mediated by plasmids related to pSS50. Appl. Environ. Microbiol. **58:**399-402.

Leahy, J.G., and R. H. Olson. 1994. 94th General Meeting Amer. Soc. Microbiol., May 23-27. Las Vegas, NV., p.307.

Li, S., and L.P. Wackett. 1992. Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Commun. **185:**443-451.

Lloyd-Jones, G., C. de Jong, R.C. Ogden, W.A. Duetz, and P.A. Williams. 1994. Recombination of the *bph* (biphenyl) catabolic genes from plasmid pWW100 and their deletion during growth on benzoate. Appl. Environ. Microbiol. **60:**691-696.

Loper, J.E., and S.E. Lindow. 1994. A biological sensor for iron available to bacteria in their habitats on plant surfaces. Appl. Environ. Microbiol. **60:**1934-1941.

Maniatis, R., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold spring Harbor, NY

Maron, D.M., and B.N. Ames. 1983. Revised methods for the *Salmonella* mutagenicity test. Mutation Res. **113:**173-215.

McTavish, H., J.A. Fuchs, and A.B. Hooper. 1993. Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. **175:**2436-2444.

Menn, F.-M., B.M. Applegate, and G.S. Sayler. 1993. NAH plasmid-mediated catabolism of anthracene and phenanthrene to naphthoic acids. Appl. Environ. Microbiol. **59:**1938-1942.

Menn, F.M., G.J. Zylstra, and D.T. Gibson. 1991. Location and sequence of the *todF* gene encoding 2-hydroxy-6-oxohepta-2,4-dienote hydrolase in *Pseudomonas putida* F1. Gene **104:**91-94.

Meynell, G., and E. Meynell. 1970. Theory and Practice in Experimental Bacteriology. Cambridge University Press, New York, p.38.

Microbics reference # D006. The mode of action of genotoxic agents in the restoration of light in the MutatoxTM system. Microbics Corporation, 2232 Rutherford Rd, Carlsbad, CA.

Mondello, F.J. 1989. Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. **171:**1725-1732.

Monticello, D.J., D. Bakker, and W.R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. **49:**756-760.

Nadeau, L.J., F.-M. Menn, A. Breen, and G.S. Sayler. 1994. Aerobic degradation of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) by *Alcaligenes eutrophus* A5. Appl. Environ. Microbiol. **60:**51-55.

Nakatsu, C.H., and R.C. Wyndham. 1993. Cloning and expression of the transposable chlorobenzoate-3,4-dioxygenase genes of *Alcaligenes* sp. strain BR60. Appl. Environ. Microbiol. **59:**3625-3633.

Natarajan, M.R., Z. Lu, and P. Oriel. 1994. Cloning and expression of a pathway for benzene and toluene from *Bacillus stearothermophilus*. Biodegradation. **5:**77-82.

Neidle, E.L., C. Hartnett, S. Bonitz, and L.N. Ornston. 1988. DNA sequence of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase I structural gene *catA*: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. J. Bacteriol. **170:**4874-4880.

Neidle, E.L., M.K. Shapiro, and L.N. Ornston. 1987. Cloning and expression in *Escherichia coli* of *Acinetobacter calcoaceticus* genes for benzoate degradation. J. Bacteriol. **169:**5496-5503.

Nelson, M.J.K., S.O. Montgomery, W.R. Mahaffey, and P.H. Pritchard. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53:**949-954.

Nelson, M.J.K., S.O. Montgomery, and P.H. Pritchard. 1988. Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. Appl. Environ. Microbiol. **54:**604-606.
Oldenhuis, R., and D.B. Janssen. 1993. Microbial growth on C_1 compounds. Andover, UK. Intercept Ltd., p.121-133.

Oldenhuis, R., R.L.J.M. Vink, D.B. Janssen, and B. Witholt. 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. **55:**2819-2826.

Olsen. R.H., J.J. Kukor, and B. Kaphammer. 1994. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J. Bacteriol. **176:**3749-3756.
Parales, R.E., and C.S. Harwood. 1993. Regulation of the *pcaIJ* genes for aromatic acid degradation in *Pseudomonas putida*. J. Bacteriol. **175:**5829-5838.

Peloquin, L., and C.W. Greer. 1993. Cloning and expression of the polychlorinated biphenyl-degrading gene cluster from *Arthrobacter* M5 and comparison to analogous genes from Gram-negative bacteria. Gene **125:**35-40.

Perkins, E.J., M.P. Gordon, O. Caceres, and P.F. Lurquin. 1990. Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J. Bacteriol. **172:**2351-2359.

Pettigrew, C.A., A. Breen, C. Corcoran, and G.S. Sayler. 1990. Chlorinated biphenyl mineralization by individual populations and consortia of freshwater bacteria. Appl. Environ. Microbiol. **56:**2036-2045.

Pieper, D.H., K.-H. Engesser, and H.-J. Knackmuss. 1989. Regulation of catabolic pathways of phenoxyacetic acids and phenols in *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **151:**365-371.

Powlowski, J., and V. Shingler. 1994. Genetics and biochemistry of phenol degradation by *Pseudomonas* sp. CF600. Biodegradation **5:**219-236.

Pries, F., J.R. van der Ploeg, A.J. van den Wijngaard, R. Bos, and D.B. Janssen. 1994. Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds. Boca Raton, FL. Lewis Publishers, p.259-265.

Reineke, W., and H.J. Knackmuss. 1988. Microbial degradation of haloaromatics. Annu. Rev. Microbiol. **42:**263-287.

Resnick, S.M., D.S. Torok, K. Lee, J.M. Brand, and D.T. Gibson. 1994. Regiospecific and stereoselective hydroxlation of 1-indanone and 2-indanone by naphthalene dioxygenase and toluene dioxygenase. Appl. Environ. Microbiol. **60:**3323-3328.

Rochkind-Dubinsky ML., G.S. Sayler, and J.W. Blackburn. 1987. Microbiological decomposition of chlorinated aromatic compounds. New York. Marcel Dekker, Inc.

Rogowsky, P.M., T.J. Close, J.A. Chimera, J.J. Shaw, and C.I. Kado. 1987. Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. J. Bacteriol. **169:**5101-5112.

Saint, C.P., N.C. McClure, and W.A. Venables. 1990. Physical map of the aromatic amine and *m*-toluate catabolic plasmid pTDN1 in *Pseudomonas putida*: location of a unique *meta*-cleavage pathway. J. Gen. Microbiol. **136:**615-625.

Sanseverino, J., B.M. Applegate, J.M. Henry King, and G.S. Sayler. 1993a. Plasmid-mediated mineralization of naphthalene, phenanthrene, and anthracene. Appl. Environ. Microbiol. **59:**1931-1937.

Sanseverino, J., C. Werner, J. Fleming, B.M. Applegate, J.M.H. King, and G.S. Sayler. 1993b. Molecular diagnostics of polycyclic aromatic hydrocarbon biodegradation in manufactured gas plant soils. Biodegradation **4:**303-321.

Sayler G.S., and J.W. Blackburn. 1989. Biotreatment of agricultural wastewater. Boca Raton, FL. CRC Press, Inc., p.53-71.

Sayler G.S., S.W. Hooper, A.C. Layton, and J.M.H. King. 1990. Catabolic plasmids of environmental and ecological significance. Microb. Ecol. **19:**1-20.

Schell, M.A. 1990. *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. Washington, D.C. American Society for Microbiology, p.165-176.

Schmitz, A., K.H. Gartemann, J. Fiedler, E. Grund, and R. Eichenlaub. 1992. Cloning and sequence analysis of genes for dehalogenation of 4-chlorobenzoate from *Arthrobacter* sp. strain SU. Appl. Environ. Microbiol. **58:**4068-4071.

Scholten, J.D., K.-H. Chang, P.C. Babbitt, H. Charest, M. Sylvestre, and D. Dunaway-Mariano. 1991. Novel enzymic hydrolytic dehalogenation of a chlorinated aromatic. Science. **253:**182-185.

Selifonova, O., R. Burlage, and T. Barkay. 1993. Bioluminescent sensors for the detection of bioavailable Hg(II) in the environment. Appl. Environ. Microbiol. **59:**3083-3090.

Serdar, C.M., and D.T. Gibson. 1989. Isolation and characterization of altered plasmids in mutant strains of *Pseudomonas putida* NCIB9816. Biochem. Biophys. Res. Commun. **164:**764-771.

Shanley, M.S., E.L. Neidle, R.E. Parales, and L.N. Ornston. 1986. Cloning and expression of *Acinetobacter calcoaceticus catBCDE* genes in *Pseudomonas putida* and *Escherichia coli*. J. Bacteriol. **165:**557-563.

Sharma, A., C.D. Chunn, R.K. Rothmel, and R. Unterman. 1991. 91st General Meeting, Amer. Soc. Microbiol., May 5-9. Dallas, TX. p.284.

Shaw J.J., L.G. Settles, and C.I. Kado. 1988. Transposon *Tn4431* mutagenesis of *Xanthomonas campestris* pv. *campestris*: characterization of a nonpathogenic mutant and cloning of locus for pathogenicity. Mol. Plant-Microbe Interact. **1:**39-45.

Shields, M.S., S.W. Hooper, and G.S. Sayler. 1985. Plasmid-mediated mineralization of 4-chlorobiphenyl. J. Bacteriol. **163:**882-889.

Shields, M.S., S.O. Montgomery, S.M. Cuskey, P.J. Chapman, and P.H. Pritchard. 1991. Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl. Environ. Microbiol. **57:**1935-1941.

Shields, M.S., and M.J. Reagin. 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol. **58:**3977-3983.

Shingler, V., J. Powlowski, and U. Marklund. 1992. Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. J. Bacteriol. **174:**711-724.

Singleton, I. 1994. Microbial metabolism of xenobiotics: fundamental and applied research. J. Chem. Tech. Biotechnol. **59:**9-23.

Skoog, D.A., and D.M. West. 1986. The evaluation of analytical data. *In* Analytical chemistry: an introduction. Saunders College Publishing, New York, p.56.

Smith, M.R. 1990. The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation **1:**191-206.

Spain, J. C. 1990. Metabolic pathways for biodegradation of chlorobenzenes, p. 197-206. *In* S. Silver; A. M. Chakrabarty; B. Iglewski, and S. Kaplan (eds.), *Pseudomonas*: Biotransformations, Pathogenesis, and Evolving Biotechnology. American Society for Microbiology, Washington, D.C.

Spain, J.C., and S.F. Nishino. 1987. Degradation of 1,4- dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. **53**:1010-1019.

Springael, D., W. Baeyens, L. Hannes, K. De Wilde, and M. Mergeay. 1991. Use of *Tn4431* containing the promoter-less *lux* genes of *Vibrio fischeri* to detect genes involved in PCB degradation of *Alcaligenes* sp. A5. Arch. Int. Physiol. Biochim. **99:**77.

Springael, D, S. Kreps, and M. Mergeay. 1993. Identification of a catabolic transposon, Tn*4317*, carrying biphenyl and 4-chlorobiphenyl degradation genes in *Alcaligenes eutrophus* A5. J. Bacteriol. **175:**1674-1681.

Strubel, V., K.-H. Engesser, P. Fischer, and H.K. Knackmuss. 1991. 3-(2-Hydroxyphenyl)catechol as substrate for proximal *meta* ring cleavage in dibenzofuran degradation by *Brevibacterium* sp. strain DPO 1361. J. Bacteriol. **173:**1932-1937.

Taira, K., N. Hayase, N. Arimura, S. Yamashita, T. Miyazaki, and K. Furukawa. 1988. Cloning and nucleotide sequence of 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain *Pseudomonas paucimobilis* Q1. Biochemistry **27:**3990-3996.

Taira, K., J. Hirose, S. Hayashida, and K. Furukawa. 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Bacteriol. **267:**4844-4853.

Tan, H.-M., and K.P.Y. Fong. 1993b. Molecular analysis of the plasmid-borne *bed* gene cluster from *Pseudomonas putida* ML2 and cloning of the *cis*-benzene dihydrodiol dehydrogenase. Can. J. Microbiol. **39:**357-362.

Tan, H.-M., and J.R. Mason. 1990. Cloning and expression of the plasmid-encoded benzene dioxygenase genes from *Pseudomonas putida* ML2. FEMS Microbiol. Lett. **72:**259-264.

Tan, H.-M., H.-V. Tang, C.L. Joannou, N.H. Abdel-Wahab, and J.R. Mason. 1993a. The *Pseudomonas putida* ML2 plasmid-encoded genes for benzene dioxygenase are unusual in codon usage and low in G+C content. Gene **130:**33-39.

Tardif, G., C.W. Greer, D. Labbe, and P.C.K. Lau. 1991. Involvement of a large plasmid in the degradation of 1,2-dichloroethane by *Xanthobacter autotrophicus*. Appl. Environ. Microbiol. **57:**1853-1857.

Timmis, K.N., P.R. Lehrbach, S. Harayama, R.H. Don, N. Mermod, S. Bas, R. Leppik, A.J. Weightman, W. Reineke, and H.J. Knackmuss. 1985. Analysis and manipulation of plasmid-encoded pathways for the catabolism of aromatic compounds by soil bacteria. *In* Plasmid in bacteria. New York. Plenum Press, p.719-739.

Tsien, H.-C., G.A. Brusseau, R.S. Hanson, and L.P. Wackett. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **55:**3155-3161.

van der Meer JR, A.R.W. van Neerven, E.J. de Vries, W.M. de Vos, and A.J.B. Zehnder. 1991. Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. J. Bacteriol. **173:**6-15.

van der Meer, J.R., W. de Vos, S. Harayama, and A.J.B. Zehnder. 1992. Molecular mechanisms of genetics adaptation to xenobiotics compounds. Microbiol. Rev. **56:**677-694.
Van Rolleghem, P., D. Drues, and W. Verstraete. 1990. Proceeding of 5th European Congress on Biotechnology, July 8-13, Copenhagen, Denmark, p. 161-164.

Wackett, L.P., and D.T. Gibson. 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703-1708.

Wallace, W.H., and G.S. Sayler. 1992. Encyclopedia of Microbiology. Vol. 1. New York. Academic Press, Inc. p.417-430.

Webb, O.F., T.J. Phelps, P.R. Bienkowski, P.M. DiGrazia, G.D. Reed, B. Applegate, D.C. White, and G.S. Sayler. 1991. Development of a differential volume reactor system for soil biodegradation studies. Appl. Biochem. Biotech. **28/29:**5-19.

Williams, P.A., and K. Murray. 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of new TOL plasmids. J. Bacteriol. **120:**416-423.

Williams, P.A., and J.R. Sayers. 1994. The evolution of pathways for aromatic hydrocarbon oxidation in *Pseudomonas*. Biodegradation **5:**195-217.

Winstanley, C., S.C. Taylor, and P.A. Williams. 1987. pWW174: a large plasmid from *Acinetobacter calcoaceticus* encoding benzene catabolism by the β-ketoadipate pathway. Mol. Microbiol. **1:**219-227.

Winter, R.B., K.-M. Yen, and B.D. Ensley. 1989. Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. Bio/Technology **7:**282-285.

Worsey, M.J., and P.A. Williams. 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124:**7-13.

Yabuuchi, E., I. Yano, H. Oyaizu, Y. Hashimoto, T. Ezaki, and H. Yamamoto. 1990. Proposal of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhesiva* sp. nov., *Sphingomonas capsulata* com. nov., and two genospecies of the genus *Sphingomonas*. Microb. Immunol. **34:**99-119.

Yano, K., and T. Nishi. 1980. pKJ1, a naturally occurring conjugative plasmid coding for toluene degradation and resistance to streptomycin and sulfonamides. J. Bacteriol. **143:**552-560.

Yates, J.R., and F.J. Mondello. 1989. Sequence similarities in the encoding polychlorinated biphenyl degradation by *Pseudomonas* strain LB400 and *Alcaligenes eutrophus* H850. J. Bacteriol. **171:**1733-1735.

Yen, K.-M., and I.C. Gunsalus. 1982. Plasmid gene organization: naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. USA. **79:**874-878.

Yen, K.-M., and I.C. Gunsalus. 1985. Regulation of naphthalene catabolic genes of plasmid NAH7. J. Bacteriol. **162:**1008-1013.

Yen, K.-M., M.R. Karl, L.M. Blatt, M.J. Simon, R.B. Winter, P.R. Fausset, H.-S. Lu, A.A. Harcourt, and K.-K. Chen. 1991. Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. J. Bacteriol. **173:**5315-5327.

Yen, K.-M., and C.M. Serdar. 1988. Genetics of naphthalene catabolism in pseudomonads. Crit. Rev. Microbiol. **15:**247-268.

Zylstra, G.J., and D.T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. **264:**14940-14946.

Zylstra, G.J., W.R. McCombie, D.T. Gibson, and B. Finette. 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the *tod* operon. Appl. Environ. Microbiol. **54:**1498-1503.

Zylstra, G.J., L.P. Wackett, and D.T. Gibson. 1989. Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. Appl. Environ. Microbiol. **55:**3162-3166.

6. PUBLICATIONS (1992 - 1995).

Applegate, B., U. Matrubutham, J. Sanseverino, and G.S. Sayler. 1994. Biodegradation genes as marker genes in microbial ecosystems. *In*: Molecular microbial ecology manual (A.D.L. Akkermans, J.D. van Elsas and F.J. de Bruijn, eds), Kluwer, Netherlands. In review.

Atlas, R.M., G.S. Sayler, R.S. Burlage and A.K. Bej. 1992. Molecular approaches for environmental monitoring of microorganisms. BioTechniques **12:**706-717.

Burlage, R.S., A. Heitzer and P.M. DiGrazia. 1992. Regulated bioluminescence as a tool for bioremediation monitoring and control of bacterial cultures. In Technology 2001, Proceedings of the second national technology transfer conference and exposition, Dec. 3-5, 1991, San Jose, CA. NASA Conference Publication 3136, Vol. I, p.292-299.

Burlage, R.S., A. Heitzer and G.S. Sayler. 1992. Bioluminescence: A versatile bioreporter for monitoring bacterial activity. Biotech Forum Europe **9:**704-709.

Burlage, R.S., A.V. Palumbo, A. Heitzer, and G.S. Sayler. 1994. Bioluminescent reporter bacteria detect contaminants in soil samples. Appl. Biochem. Biotechnol. **45/46:**731-740.

Fleming, J.T., and G.S. Sayler. 1994. Extraction of RNA from bulk soils. *In*: Molecular microbial ecology manual. (A.D.L. Akkermans, J.D. van Elsas and F.J. de Bruijn, eds), Kluwer, Netherlands. In review.

Fleming, J.T., and G.S. Sayler. 1995. Assessment of gene expression in the environment: quantitative mRNA analysis. in contaminated soils. *In*: Applications of molecular biology in environmental chemistry. (R.A Minear, A. Ford, L. Needham, and N. Karch eds), CRC Press, p.45-58.

Heitzer, A., B. Applegate, O.F. Webb, T.J. Phelps, G.S. Sayler, D.C. White. 1995. Catabolic bioluminescent reporter bacteria: physiological aspects of environmental applications. Appl. Environ. Microbiol. In review.

Heitzer, A, R.S. Burlage, P.M. DiGrazia and G.S. Sayler. 1992. Bioluminescent reporters for catabolic gene expression and pollutant bioavailability. *In*: Short communications of the 1991 international marine biotechnology conference, IMBC 1991, Developments in industrial microbiology extended abstract series Vol. I, p.298-306. W.C. Brown Publisher, Dubuque, Iowa.

Heitzer, A., K. Malachowsky, J.E. Thonnard, P.R. Bienkowski, D.C. White, and G.S. Sayler. 1994. Optical biosensor for environmental on-line monitoring of naphthalene and salicylate bioavailability using an immobilized bioluminescent catabolic reporter bacterium. Appl. Environ. Microbiol. **60:**1487-1494.

Heitzer, A. and G.S. Sayler. 1994. Monitoring the Efficacy of Bioremediation. Trends in Biotechnology **8:**334-343.

Heitzer, A., O.F. Webb, J.E. Thonnard and G.S. Sayler. 1992. Specific and quantitative assessment of naphthalene and salicylate bioavailability by using a bioluminescent catabolic reporter bacterium. Appl. Environ. Microbiol. **58:**1839-1846.

Heitzer, A., O.F. Webb, P.M. DiGrazia and G.S. Sayler. 1995. A versatile bioluminescent reporter system for organic pollutant bioavailability and biodegradation. *In*: Applications of molecular biology in environmental chemistry (R.A Minear, A. Ford, L. Needham, and N. Karch eds.). CRC Press, p.191-208.

Heitzer, A., O.F. Webb, P.M. DiGrazia and G.S. sayler (1992). A versatile bioluminescent reporter system for organic pollutant bioavailability and biodegradation. Proceedings of the American Chemistry Society meeting, Division of Environmental Chemistry, Aug. 23-28, Washington D.C.

Johnston, W.H., R. Stapleton, and G.S. Sayler. 1994. Direct extraction of microbial DNA from soils and sediments. *In*: Molecular microbial ecology manual (A.D.L. Akkermans, J.D. van Elsas and F.J. de Bruijn, eds), Kluwer, Netherlands. In review.

Layton, A.C., C.A. Lajoie, J.P. Easter, R. Jernigan, M.J. Beck and G.S. Sayler. 1994. Molecular diagnostics for polychlorinated biphenyl degradation in contaminated soils. *In*: Recombinant DNA Technology II, Vol. 721, Annals of the New York Academy of Sciences. p. 407-422.

Layton, A.C., J. Sanseverino, W. Wallace, C. Corcoran and G.S. Sayler. 1992. Evidence for 4-chlorobenzoic acid dehalogenation mediated by pSS50 related plasmids. Appl. Environ. Microbiol. **58:**3012-3019.

Menn, F.-M., B.M. Applegate and G.S. Sayler. 1993. NAH plasmid mediated catabolism of anthracene and phenanthrene to naphthoic acids. Appl. Environ. Microbiol. **59:**1938-1942.

Nadeau, L., F.-M. Menn, A. Breen and G.S. Sayler. 1994. Aerobic degradation of 1,1,1-trichloro-2,2-bis (4-chlorophenyl)ethane (DDT) by *Alcaligenes eutrophus* A5. Appl. Environ. Microbiol. **60:**51-55.

Pullen, R., G.D. Reed, and G.S. Sayler. 1995. Using bioluminescence assays to monitor microbial degradative activity in soil- or sediment-containing systems. J. Indust. Microbiol. In review.

Sanseverino, J., B.M. Applegate, J.M. Henry King and G.S. Sayler. 1993. Plasmid-mediated mineralization of naphthalene, phenanthrene and anthracene. Appl. Environ. Microbiol. **59:**1931-1937.

Sanseverino, J., J.T. Fleming, A. Heitzer, B.M. Applegate, and G.S. Sayler. 1994. Applications of environmental biotechnology to bioremediation. *In*: Remediation of hazardous waste contaminated soils (D. Wise and D. Trantolo, eds.) Marcel Dekker, Inc., p.97-123.

Sanseverino, J., C. Werner, J.T. Fleming, B.M. Applegate, J.M.H. King, and G.S. Sayler. 1993. Molecular diagnostics of polycyclic armoatic hydrocarbon biodegradation in manufactured gas plant soils. Biodegradation **4**:303-321.

Sayler, G.S. 1993. Environmental biotechnology's furture in hazardous waste management. *In*: Biotechnology: Theory and applications (W. Tien, L. Lo, S. chen, Y. Shuy, eds), p.248-254.

Sayler, G.S., J.T. Fleming, B. Applegate and C. Werner. 1992. Nucleic acid extraction and analysis: Detecting genes and their activity in the environment. In: Genetic interactions between microorganisms in the natural environment (E.M. Wellington and J.D. Van Elsas, eds), Pergamon Press, p.237-257.

Sayler, G.S., U. Matrubutham, F.-M. Menn, W. Johnston, and R. Stapleton. 1995. Molecular probes and biosensors in bioremediation and site assessment. *In*: (S.K. Sikdar and R.L. Irvine, eds) Bioremediation - Principle and practice. Technomic Publishing Co. Inc.

Sayler, G.S., K. Nikbakht, J. Fleming and J. Packard. 1992. Application of molecular techniques in soil biotechnology. *In*: Soil biochemistry (G. Stotzky and J. Bollag, eds) Vol.7, pp.131-162.

Strong-Gunderson, J.M., B. Applegate, G.S. Sayler, and A.V. Palumbo. 1994. Biosurfactants and Increased bioavailability of sorbed organic contaminants: measurements using a biosensor. (IGT Proceedings), In review.

Wallace, W., and G.S. Sayler (1992). Plasmids in the environment. In: Encyclopedia of Microbiology, Vol.1. J. Leberberg (ed), p.417-430, Academic Press.

7. PRESENTATIONS AND ABSTRACTS

Applegate, B., L. Lackey, J. McPherson and F.-M. Menn (1993). A bioluminescent reporter for the co-oxidation of trichloroethylene (TCE) by the toluene dioxygenase in *Pseudomonas putida* F1. Abstract Q-108, p365. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Applegate, B., J. McPherson, F.-M. Menn, A. Heitzer and G.S. Sayler (1992). Application of bioluminescent reporter technology as a tool to investigate the involvement of the Nah system in the catabolism of different polyaromatic hydrocarbons. Abstract Q-64, p.346. American Society for Microbiology (ASM) Annual Meeting, New Orleans, LA.

Callicotte, L., J. Sanseverino, B. Applegate and F.-M. Menn (1992). Preliminary characterization of the naphthalene catabolic pathway of *Pseudomonas* sp. JS1. Abstract Q-66, p.346. American Society for Microbiology (ASM) Annual Meeting, New Orleans, LA.

Heitzer, A., J.E. Thonnard and G.S. Sayler (1993). Continuous on-line pollutant monitoring using a whole cell biosensor based on a bioluminescent catabolic reporter bacterium. Abstract Q-97, p364. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Heitzer, A., O.F. Webb, P.M. DiGrazia and G.S. Sayler (1992). A versatile bioluminescent reporter system for organic pollutant bioavailability and biodegradation. American Society for Microbiology (ASM) Annual Meeting, New Orleans, LA.

Heitzer, A., O.F. Webb and G.S. Sayler (1992). Specific and quantitative assessment of naphthalene and salicyalte bioavailability using a bioluminescent catabolic reporter bacterium. American Society for Microbiology (ASM) Annual Meeting, New Orleans, LA.

Huang, B., T.W. Wang, R. Burlage and G.S. Sayler (1992). Development of an on-line sensor for bioreactor operation. Proceeding of the Fourteenth Symposium on Biotechnology for Fuels and Chemicals.

Johnston, W. and G.S. Sayler (1992). Maintenance and stability of nah-lux bioluminescent reporter strains and plasmids. American Society for Microbiology (ASM) Annual Meeting, New Orleans, LA.

Menn, F.-M., J. Sanseverino, B. Applegate and G. Sayler (1993). NAH plasmid mediated catabolism of polycyclic aromatic hydrocarbons. Abstract Q-348, p.122. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Nadeau, L., F.-M. Menn, A. Breen and G. Sayler (1993). The aerobic degradation of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) by *Alcaligenes eutrophus*. Abstract Q-158, p.51. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Sanseverino, J., C. Werner, J. Fleming, B.M. Applegate, J.M. Henry King and G.S. Sayler (1993). Molecular diagnostic of polycyclic aromatic hydrocarbon biodegradation in manufactured gas plant soils. Abstract Q-349, p.410. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Sayler, G.S. (1992). Environmental biotechnology's future in hazardous waste management. Third Pacific Rim Biotechnology Conference, Taipei, Taiwan.

Sayler, G.S. (1992). Bioluminescent monitoring of degradative gene expression in soils. American Society of Agronomy. Minneapolis, Minnesota.

Sayler, G.S. (1992). Molecular approaches for diagnostics, performance monitoring and optimization in bioremediation. U.S./Israel Bioremediation Workshop, Tel Aviv, Israel.

8. LECTURES AND SEMINARS (G.S. Sayler)

(1994)

University of South Carolina, "Molecular Technology in Hazardous Waste Site Characterization and Bioremediation", Columbia, SC. Invited seminar.

Universidad Interamericana de Puerto Rico, "Integrating Molecular Biology in Environmental Analysis: Hazardous Waste Site Characterization and Remediation", San Juan, Puerto Rico. Invited Speaker.

Vanderbilt University, "Molecular Approaches in Hazardous Waste Biodegradation Research", Nashville, TN. Invited Seminar.

Engelbrecht Symposium, "Molecular Probes and Biosensors for Bioremediation Process Monitoring and Control", Urbana, IL. Invited Presentation.

XVIII Congress of Scientific Research, "Bioluminescent Reporter Technology: Genetic Engineering for Chemical and Microbial Process Sensing", San German, Puerto Rico. Invited Presentation.

University of Nebraska Biotechnology Seminar, "Molecular Probes and Biosensors in Bioremediation Process Monitoring and Optimization", Lincoln, NE. Invited Speaker.

University of Minnesota Microbial Ecology Minors Program Seminar Series, "Molecular Approaches in Assessing Soil and Subsurface Soil Hazardous Waste Remediation", St. Paul, MN. Invited Seminar.

American Society for Microbiology Annual Meeting, "PAH Transformations", Las Vegas, NV. Invited Presentation.

JASON Summer Experience, Bioremediation 101, "Molecular Diagnostics: Probes and Reporters in Bioremediation Process Monitoring and Control", U.S. DOE, LaJolla, CA. Invited Presentation.

7th International Symposium on the Genetics of Industrial Microorganisms (GIM94), "Bioluminescent Reporter Fusions to Monitor Organic Chemical Biodegradation", Montreal Canada. Invited Presentation.

GIM94 Satellite Workshop: Plasmid Diversity, "Plasmids Associated with PAH Biodegradation", Montreal Canada. Invited Presentation.

DOE Fallen Leaf Lake Conference, "Biodegradative Gene Abundance and Expression in Contaminated Environments", South Lake Tahoe, CA. Invited Speaker.

7th Annual Colorado Biotechnology Symposium, "Molecular Environmental Diagnostics of Biodegradative Microbial Community Structure and Activity", Denver, CO. Invited Speaker.

Miami University, "Molecular Environmental Diagnostics in Biodegradation Research and Bioremediation", Oxford, OH. Distinguished Lecturer.

FASEB, Joint U.S.-European Commission Workshop on Environmental Biotechnology, "Biodegradation Process Analysis: Molecular Application in Simulations and Environmental Verification", Brussels, Belgium. Invited Lecture.

Rhone-Poulenc, Meeting on Biodegradability and Formulation, "Genetic and molecular models in biodegradation assessment and monitoring", Research Triangle Park, NC. Invited Lecture.

RTDF Workshop on Microbial Characterization of *In Situ* Chlorinated Solvent Biodegradation, Cincinnati, OH, Invited Lecture.

ASM Foundation Lecture, Georgia-South Carolina Branch ASM Meeting, Athens, GA.

OECD Workshop Tokyo '94 on Bioremediation, "Reporter Genes for Monitoring Biodegradative Activities", and "Risk Assessment for Recombinant Pseudomonads Released into the Environment for Hazardous Waste Degradation", Tokyo, Japan. Invited Speaker.

(1993)

American Academy of Microbiology, Strategies and Mechanisms for Field Research in Environmental Remediation, San Antonio, TX. Invited participant and discussion leader.

Oklahoma State University, Department of Microbiology and Cell Biology, "Environmental Molecular Diagnostics", Stillwater, OK. Invited Seminar.

TOCOEN Toxic Organic Compounds in the Environment, "Molecular Technologies Applied to PCB and PAH Biodegradation Analysis", Znojmo, Czech Republic, Invited participant.

University of Washington, Department of Microbiology, "Molecular Approach in Biodegradation Assessment", Seattle, WA. Invited seminar.

NIEHS Biodegradation Workshop, "Molecular Approaches for Biodegradation Assessment", Triangle Park, NC. Invited seminar.

American Society of Microbiology Annual Meeting, "Application of Gene Fusions in Monitoring Gene Expression in Degradative Bacteria *In situ*, Atlanta, GA., Invited speaker.

Celgene Corporation, "Microbial and Molecular Monitoring of Biodegradation Processes", Warren, NJ, Invited seminar.

Keystone Molecular Biology Symposium, Environmental Bioremediation and Biodegradation, "Molecular Strategies in Biodegradation Process Monitoring and Optimization" Tahoe City, CA., Invited participant.

American Chemical Society Southeast Regional Meeting, "Bioluminescent Reporter Technology: Genetic Engineering for Chemical and Microbial Process Sensing" Johnson City, TN Invited Presentation.

Environmental Protection Agency, Frontiers in Bioprocessing III, "Molecular Site Assessment and Process Monitoring in Bioremediation and Natural Attenuation", Boulder, CO, Invited Lecturer.

Chilean Association of Microbiology, Symposium on Biodegradation of Industrial Wastes and Pesticides, "Application of Molecular Biology in Measuring Microbial Biodegradation of Organic Pollutants", Santiago, Chile, Invited Participant.

ONR/NRL Environmental Quality Seminar Series, "Lux-Gene Fusions: Bioluminescent Reports for Environmental Biodegradation and Biosynthesis", Arlington, VA, Invited Lecturer.

(1992)

Virginia Tech and State University, Department of Biology, Blacksburg, VA. Invited seminar.

Sigma XI - Southern Appalachia Regional Lecture, ETSU, Johnson City, TN. Invited lecture.

University of North Carolina Charlotte, Department of Biology, Environmental Biotechnology Lecture. Invited lecture.

Rensselaer Polytechnic Institute, Department of Biology, Troy, NY. Invited Bray lecture.

EPA Cincinnati, Environmental Research Laboratory, Biotechnology Lecture, Invited lecture.

Third Pacific Rim Biotechnology Conference, Taipei, Taiwan. Invited lecture.

American Society of Agronomy. Minneapolis, Minnesota. Invited lecture.

U.S./Israel Bioremediation Workshop, Tel Aviv, Israel. Invited lecture.